Expression of CSI and 2B4 in Human Lupus Erythematosus and Transcriptional Regulation of CSI Gene

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ABSTRACT

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by production of pathogenic autoantibodies that affect multiple organs. Signaling lymphocyte activation molecule (SLAM) family receptors play critical roles in the regulation of hematopoietic cells, and polymorphisms in these receptors were found to be associated with susceptibility to SLE. In addition, the differential expression of splice variants of one member of SLAM family receptors was shown to be responsible for lupus development in mice. Since the important roles of SLAM family receptors 2B4 and CS1 in immune regulation are expanding, we compared the expression of 2B4 and CS1 in peripheral blood mononuclear cells (PBMCs) from SLE patients with those of healthy individuals. While alterations of the expression of 2B4 and CS1 were observed in SLE, most strikingly, a linear relationship was found between the proportion of CS1-expressing B cells and SLE disease activity index (SLEDAI). In particular, CS1-high expressing B cells were phenotypically similar with recently described plasmablasts, which were also shown to correlate with disease severity. Since CS1 is self-ligand and homotypic interaction of CS1-expressing B cells can trigger expansion of B cells, these results emphasize the role of CS1 in B cell-mediated diseases. Therefore, the transcriptional regulation of CS1 gene was investigated. DNA elements at -405 to +92 region of the CS1 promoter positively regulated CS1 expression. Interestingly, electrophoretic mobility shift and chromatin immunoprecipitation assays revealed that the plasma cell specific-transcription factor Blimp-1 binds to the CS1 promoter region.
Further analysis of expression and transcriptional regulation of CS1 in B cells will provide valuable information for the treatment of B-cell mediated diseases such as SLE and multiple myeloma.
EXPRESSION OF CS1 AND 2B4 IN HUMAN SYTEMIC LUPUS ERYTHEMATOSUS AND TRANSCRIPTIONAL REGULATION OF CS1 GENE

DISSERTATION

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Jong Rok Kim, M.S.
Fort Worth, Texas
July 2010
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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The Immune System

The immune system is a host security system that protects an organism from foreign pathogens and tumor cells. It is classically divided into two functional arms: innate immunity and adaptive immunity. Innate immunity is present at birth and provides the first line of defense right after infection. Cells of the innate immune system, such as phagocytes and natural killer (NK) cells, are well equipped with pattern recognition receptors (PRRs) so that they are able to detect components called pathogen-associated molecular patterns (PAMPs) that are common to pathogens but rare in the host (Schroder and Bowie 2005; Kumar, Kawai et al. 2009). On the other hand, adaptive immunity is acquired during the life with exposure to countless foreign antigens. Cells of the adaptive immune system are T lymphocytes and B lymphocytes. Through the recombination of genes encoding T cell receptors (TCRs) and B cell receptors (BCRs), these cells can selectively recognize a specific antigen. In addition to this specificity, adaptive immunity has another important feature, i.e. immunological memory. Generation of long-term memory T and B cells recognizing a particular pathogen enables protective immunity against future infections. Although it is known that the innate immune system cannot give rise to memory against pathogens, recent evidence supports that NK cells are also capable of mounting a memory immune response (O’Leary, Goodarzi et al. 2006; Cooper, Elliott
et al. 2009; Sun, Beilke et al. 2009). The innate and adaptive immune systems are able to influence each other (Ehlers 2004).

The Cells of the Immune System

The cells of the immune system are often called leukocytes and they circulate through the body via blood and lymphatic vessels. Three different types of leukocytes exist – lymphocytes, monocytes, and granulocytes. Whereas granulocytes such as neutrophils, eosinophils, and basophils have multinucleated morphology, lymphocytes and monocytes display round nuclei. Lymphocytes and monocytes can be separated from granulocytes and red blood cells by density-gradient centrifugation of whole bloods, resulting in peripheral blood mononuclear cells (PBMCs). Although PBMCs may not represent the immune cells in the peripheral tissue or lymphoid organs, many studies utilize PBMCs to monitor diseases and infections because they are readily accessible.

Lymphocytes can be divided into T lymphocytes, B lymphocytes, and NK cells. T lymphocytes play a central role in cell-mediated immune responses and are classified into \( \alpha:\beta \) T cells and \( \gamma:\delta \) T cells based on the composition of their TCRs. TCRs are unique membrane receptors in that they are able to dual-recognize major histocompatibility complex (MHC) loaded with self or foreign peptides. The most common T lymphocytes are CD4\(^+\) T cells and CD8\(^+\) T cells. CD4 and CD8 are co-receptors for TCRs. CD4-TCR complexes bind to MHC class II (MHC-II) molecules expressed on antigen presenting cells (APCs), while CD8-TCR complexes bind to MHC class I (MHC-I) molecules expressed on all nucleated cells. CD8\(^+\) T cells are often named as cytotoxic T
lymphocytes (CTLs) since they are capable of killing infected target cells (MHC-restricted cell-mediated cytotoxicity). CD4$^+$ T cells are designated as helper T cells, because they provide helper signals to other immune cells by producing various cytokines, such as interferon-γ, interleukin-4, interleukin-17, interleukin-10, and tumor growth factor-β (Zhou, Chong et al. 2009).

T lymphocytes originate in the bone marrow but all the important developmental events take place in the thymus. In the thymic cortex, immature thymocytes pass two important checkpoints. First, they undergo gene rearrangement of TCR β-chain locus, and those cells with a productively rearranged β-chain are selected to differentiate from CD4$^-$ CD8$^-$ double-negative (DN) thymocytes into CD4$^+$ CD8$^+$ double-positive (DP) thymocytes (β-selection). Second, after successful rearrangement of TCR α-chain, DP thymocytes are subjected to positive selection, in which only thymocytes expressing TCRs with an intermediate affinity for self-MHC are able to survive and down-regulates either CD4 or CD8 expression to become single-positive (SP, CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$) thymocytes. In the thymic medulla, SP thymocytes responding to MHC-self peptide complexes with a high affinity, i.e. autoreactive thymocytes, are eliminated by apoptosis. This negative selection procedure serves as an important self-tolerance mechanism preventing development of autoimmunity. Mature T lymphocytes further migrate into secondary lymphoid organs such as spleen and lymph nodes, and wait for the encounter of antigenic peptides that are presented by APCs. Three types of cells can function as APCs - dendritic cells (DCs), macrophages, and B cells. Nowadays, it is known that these APCs can uptake extracellular antigens, process into small peptides, and present them
into both CD4⁺ and CD8⁺ lymphocytes; the later process is called cross-presentation (Lin, Zhan et al. 2008).

NK cells, also known as large granular lymphocytes (LGLs), are bone-marrow derived lymphocytes that play a major role in the rejection of tumors and virus-infected cells. Terminology of “Natural Killer” was used because they do not require prior sensitization to perform the cytotoxicity of target cells. Unlike B and T lymphocytes, NK cells lack antigen-specific receptors. Instead, NK cell activation and cytotoxicity are believed to be determined by a delicate balance between stimulatory and inhibitory signals received from the cell surface receptors. In particular, downregulation of surface expression of MHC-I is often regarded as a sign of infection or tumor. Although CTLs cannot perform cytotoxicity against MHC-I-deficient cells, NK cells are capable of killing them because several inhibitory receptors recognizing MHC-I do not transmit inhibitory signals. This finding provided the basis for the missing-self hypothesis about how NK cells kill the target cell. The NK cell cytotoxic activity is mainly driven by the release of small granules, which contain perforin and granzymes. Perforin forms pores in the target cell membrane, and granzymes enter through the pore and induce apoptotic cell death in target cells. Another effector function of NK cells is cytokine production, such as IFN-γ, TNF-α, and GM-CSF. Furthermore, several studies indicated that NK cells can also produce anti-inflammatory cytokine TGF-β (Gray, Hirokawa et al. 1994; Horwitz, Gray et al. 1997; Gray, Hirokawa et al. 1998; Horwitz, Gray et al. 1999; Chen, Han et al. 2009; Ghio, Contini et al. 2009). IL-2 is a well known activator for NK cell functions. In addition, activation of NK cells can be also accomplished by cytokines produced by
monocytes, called monokines, such as IL-12, IL-15, and IL-18. Human NK cells are usually identified by surface expression of CD16 (FcγRIII) and CD56. CD16 is a receptor recognizing the Fc portion of immunoglobulin (Ig) G and plays a role in antibody-dependent cellular cytotoxicity (ADCC), in which NK cells lyse the IgG-coated target cells. CD56 is a highly sialylated glycoprotein and is composed of five Ig-like domains and two fibronectin type III domains. Two different subsets of human NK cells have been reported based on surface CD56 expression level – CD56^{dim} NK cells and CD56^{bright} NK cells. Whereas CD56^{dim} NK cells are more effective at cytolysis of target cells, CD56^{bright} NK cells are more active producers of proinflammatory cytokines such as IFN-γ and TNF-α (Jacobs, Hintzen et al. 2001). In addition, CD56^{bright} NK cells predominate in lymph nodes and sites of inflammation (Fehniger, Cooper et al. 2003; Dalbeth, Gundle et al. 2004). Recent studies suggested that CD56^{bright} NK cells are precursors of CD56^{dim} NK cells, the latter having shorter telomere (Chan, Hong et al. 2007; Romagnani, Juelke et al. 2007). Interestingly, there are some reports insisting that CD56^{bright} NK cells are regulatory NK cells because they can inhibit T-cell proliferation and produce the immunosuppressive cytokine IL-10 (Li, Lim et al. 2005; Bielekova, Catalfamo et al. 2006; Deniz, Erten et al. 2008). It is noteworthy that uterine NK cells also display CD56^{bright} phenotype and play an important role in maintenance of pregnancy (Kitaya, Yasuda et al. 2003; Saito, Nakashima et al. 2008).

Monocytes, another main constituent of PBMCs, are continuously generated from monoblasts in the bone marrow. In steady state, they circulate through the bloodstream for approximately 1 to 3 days and reach the peripheral tissues for the supply of
macrophages and DCs (Kumar and Jack 2006). Monocytes can perform phagocytosis either by directly recognizing PAMPs via PRRs such as toll-like receptors (TLRs) or indirectly recognizing opsonized antigens via Fc receptors or complement receptors (Ziegler-Heitbrock 2007). Monocytes are also capable of killing infected cells by producing reactive oxygen and nitrogen species, and antimicrobial peptides (Heo, Ju et al. 2006; Rivas-Santiago, Hernandez-Pando et al. 2008). Recently, monocytes were found to directly interact with various immune cells, including T cells, B cells, NK cells, and granulocytes, modulating their functions (Pryjma, Flad et al. 1986; Halvorsen, Olsen et al. 1993; Gonzalez-Alvaro, Dominguez-Jimenez et al. 2006; Welte, Kuttruff et al. 2006; Mueller, Boix et al. 2007; Kloss, Decker et al. 2008). In addition to direct cell-to-cell interaction, monocytes can also produce pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8; anti-inflammatory cytokines such as IL-10 and TGF-β; and monokines such as IL-12, IL-15, and IL-18, which mainly activate NK cells or induce Th1 cells (Donnelly, Freeman et al. 1995; Goulart, Mineo et al. 2000; Veenstra, Jonak et al. 2002; Dalbeth, Gundle et al. 2004; Johansson, Lawson et al. 2005). Similar to NK cells, monocytes have activating and inhibitory receptors. For example, FcγRIIa acts as an activating receptor, whereas FcγRIIb acts as an inhibitory receptor (Pricop, Redecha et al. 2001; Tridandapani, Siefker et al. 2002; Wijngaarden, van de Winkel et al. 2004; Liu, Masuda et al. 2005). Recent advances distinguishing neutrophils and monocytes in mouse emphasizes the important role of monocytes in protective immune responses against several pathogens (Dunay, Damatta et al. 2008). In humans, circulating monocytes are divided into two subsets on the basis of the expression of CD14 and CD16: CD14^{hi}CD16^{-}
vs. CD14<sup>lo</sup>CD16<sup>+</sup> monocytes, which represent inflammatory and surveillance populations, respectively (Serbina, Jia et al. 2008).

**B Lymphocytes and Immunoglobulins**

Along with T lymphocytes, B lymphocytes play an essential role in adaptive immunity via their antigen-recognition molecule called immunoglobulins (Ig). Ig can exist in two forms – membrane-bound form on the B cell surface (BCR) or secreted form (antibody). Each Ig molecule has a symmetric structure composed of two identical heavy chains (H) and two identical light chains (L). This enables an antibody molecule to simultaneously recognize two identical antigenic structures. Both H and L chains contain variable (V) and constant (C) regions, which determine antigen specificity and effector functions, respectively (Schroeder and Cavacini 2010). The antigenic diversity of the Ig repertoire is generated by four main ways; three during development in bone marrow and one after encounter of antigen in germinal center. First, there are multiple copies of the germline V, D (diversity) and J (joining) gene segments, and the gene rearrangement that joins different combinations of gene segments generates a substantial part of antigenic diversity: combinatorial diversity. Second, during the recombination process, nucleotides are randomly added or removed at the joints between the different gene segments: junctional diversity. Third, another combinatorial diversity arises from the different combinations of H and L chain V regions, which combine to form the antigen-binding site in the antibody. These three processes require terminal deoxynucleotidyl transfease (TdT), and recombination-activating genes (RAG-1 and RAG-2). Finally, somatic
hypermutation (SHM) occurs at three complementarity-determining regions (CDRs) of V regions both in H and L chains, and increases the opportunity of enhanced binding to antigen. SHM process is dependent on activation-induced deaminase (AID), which deaminates dC residues in the V region of Ig genes and uracil-DNA glycosylase (UDG), which enables for the enzymatic excision or replacement (Fanning, Connor et al. 1996). Isotype class switch recombination (CSR) also occurs in the germinal center by which enables expression of IgG, IgA, or IgE in addition to IgM and IgD.

Central B Cell Development

In mammals, B lymphocytes develop throughout life in the bone marrow from the common lymphoid progenitor cells. The important feature of B cell development is that genes for Ig H and L chains are recombined in a sequential manner, suggesting that successful recombination needs to be tested at defined checkpoints before progressing to the next step of development (von Boehmer and Melchers 2010). First, signals from bone marrow stromal cells initiate the differentiation from the common lymphoid precursor cells into the progenitor B cells (pro-B cells). Pro-B cells express the essential enzymes for Ig DNA rearrangement such as RAG-1, RAG-2, and TdT. D-J joining at H chain locus takes place at the early pro-B cell stage. These cells further differentiate into the late pro-B cells that undergo V-DJ recombination. Once the productive rearrangement of H chains is finished, pro-B cells become precursor B cells (pre-B cells). Then, pre-B cells express µH chains on their membranes with surrogate light chains, which are the same on every pre-B cell. Surrogate L chains consist of VpreB and λ5 proteins, and the non-Ig
portion of λ5 plays an important role in initiating pre-BCR signaling (Bradl, Wittmann et al. 2003; Ohnishi and Melchers 2003). Pre-BCR signaling results in the prevention of second rearrangement of H chain (allelic exclusion), the downregulation of the surrogate L chain, and clonal expansion of B cells expressing the same µH chain (positive selection) (Minegishi and Conley 2001). Since dividing cells are larger than resting cells, these cells are called the large pre-B cells. This stage can be the first checkpoint for the induction of self-tolerance (Minegishi and Conley 2001; Keenan, De Riva et al. 2008; von Boehmer and Melchers 2010). After several rounds of cell division, large pre-B cells give rise to small pre-B cells, in which L chain locus V-J joining begin. The cells that have undergone a productive L chain rearrangement enter the IgM-positive immature B cell pool. Since immature B cells are very sensitive to self-antigen binding, this stage acts as a second checkpoint which is critical for the induction of self-tolerance (Herzog, Reth et al. 2009). After this negative selection checkpoint, immature B cells leave the bone marrow and immigrate to the spleen as transitional B cells to continue their maturation process (von Boehmer and Melchers 2010).

**Checkpoints for Polyreactive B cells**

The main dogma in immunology is ‘one antibody, one antigen binding specificity (Zhou, Tzioufas et al. 2007). However, many BCRs from developing B cells do not follow this rule, and have polyreactivity; i.e. they are able to bind multiple antigens, such as single-stranded DNA, double-stranded DNA, insulin, and lipopolysaccharides (LPS), regardless of their foreignness. For example, at the early immature B cell stage, where
complete IgM is not yet expressed on the surface, 55% of all Ig repertoires are polyreactive (Wardemann, Yurasov et al. 2003). The striking feature of polyreactive antibody is that V regions of IgH chain have CDR3 regions that contain positively charged and aromatic amino acid residues (Palanichamy, Barnard et al. 2009). Interestingly, the non-Ig portion of λ5 in the pre-BCR complex also has positively charged arginine residues and is polyreactive against multiple structures, including DNA, insulin, and LPS (Bradl, Wittmann et al. 2003; Ohnishi and Melchers 2003; Kohler, Hug et al. 2008). Since IgH chain and λ5 are components of pre-BCR, it is thought that the polyreactivity of pre-BCR has some beneficial effects on pre-B cells. Recently, it was suggested that once expressed on the surface, single pre-BCR molecules are immediately aggregated for signaling because of the polyreactive nature of pre-BCRs (Herzog, Reth et al. 2009). Therefore, although it has been generally regarded that the transition from pro-B cells to pre-B cells is antigen-independent process, the polyreactivity of pre-BCR suggests that this first checkpoint also involves soluble or membrane-bound self-antigens for the positive or negative selection of pre-B cells (Minegishi and Conley 2001; Keenan, De Riva et al. 2008; Kohler, Hug et al. 2008; von Boehmer and Melchers 2010).

**Checkpoints for Autoreactive B Cells**

Since the most important hallmark of the immune system is to distinguish between self and nonself, the acquisition of self-tolerance is critically important during the lymphocyte development. In fact, it is estimated that 75% of all newly generated BCRs are autoreactive. This number gradually decreases as the B cells mature, indicating
that self-tolerance mechanisms are in place to control autoreactive B cells (Wardemann, Yurasov et al. 2003). Three mechanisms are known to be involved in silencing autoreactive antibodies: receptor editing, clonal deletion, and B cell anergy (Kumar, Li et al. 2006). Receptor editing is a unique mechanism for developing B cells to replace their Ig receptors. Immature B cells are the main cells that undergo receptor editing, because they can have four opportunities to rearrange IgL chains from \( \kappa \) or \( \lambda \) loci in maternal or paternal chromosomes. If immature B cells express high levels of non-autoreactive receptors because the IgH and IgL chains pair well, then they will receive the right amount of BCR signaling, resulting in the termination of RAG expression and the progression into the next stage (positive selection). On the other hand, if immature B cells express low levels of non-autoreactive receptors because the IgH and IgL chains poorly interact, the signaling via the receptor is below the threshold required for positive selection. In this case, immature B cells do not turn off RAG expression and are able to undergo secondary IgL chain rearrangements. Similarly, if immature B cells express autoreactive receptors, strong crosslinking of surface IgM by self antigen halts further development and RAG expression will be continued, leading to the rearrangement of IgL gene segments. This process leads to a change in the specificity of a previously autoreactive BCR. If the resulting new BCR is non-autoreactive and the new IgL chain pairs well with IgH chain, these immature B cells will be positively selected for further development. When the new BCRs also fail to eliminate autoreactivity, the cell undergoes apoptosis. This mechanism is known as clonal deletion. In addition, when cells bind lower avidity antigens, they become anergic, a state in which they undergo intrinsic
biochemical changes that make them resistant to activation and less able to compete for survival factors. Whereas clonal deletion and receptor editing confer the central B cell tolerance, B cell anergy usually takes place in the periphery (Meffre and Wardemann 2008; von Boehmer and Melchers 2010).

**Transitional B Cells**

Transitional B cells can be further subdivided into three populations. B cells initially transitioning from the bone marrow to the spleen are referred to as transitional type 1 (T1) cells, and have $CD21^{lo} CD23^{lo} IgD^{lo}$ phenotype. Upon maturation to the next stage of transitional type 2 (T2) cells, all three surface molecules are upregulated ($CD21^{hi} CD23^{hi} IgD^{hi}$) along with CD62L, a homing receptor for entering secondary lymphoid tissues. Transitional type 3 (T3) cells downregulate their surface IgM expression while maintaining high level of surface IgD (Duong, Ota *et al.* 2010). Although the surface phenotype between T3 cells and mature naïve B cells are very similar, a useful marker to distinguish them is the ABCB1 transporter that is only expressed on mature naïve B cells (Palanichamy, Barnard *et al.* 2009). Several *in vitro* and *in vivo* studies have shown that the transitional stage of B cell development is yet another checkpoint for BCR autoreactivity (Petro, Gerstein *et al.* 2002; Su and Rawlings 2002; Meffre and Wardemann 2008). Furthermore, the finding of IL-10-producing regulatory B cells within T2 population increases the importance of this stage of B cells in immune regulation (Evans, Chavez-Rueda *et al.* 2007; Blair, Norena *et al.* 2010).
Peripheral B Cell Lineages

Peripheral B cells are generally divided into B-1 cells and the conventional B-2 cells. B-1 cells, constituting 1 to 5% of total B cells, are present in low numbers in the spleen and intestine, but are predominantly found in the peritoneal and pleural cavities (Duong, Ota et al. 2010). The distinctive phenotype of B-1 cells is expression of higher levels of surface IgM and lower levels of surface IgD. Although most B-1 cells also express CD5, a minority subset of B-1 cells display CD5-negative phenotype; CD5+ B1 cells are called B-1a whereas CD5− B1 cells are called B-1b cells (Duan and Morel 2006; Alugupalli 2008). B-1 cells develop during the prenatal period in the fetal liver and persist after birth by self-renewal. It is known that B-1 cells do not develop into memory B cells. B1 BCR is much less diverse than that of B-2 cells because of their restricted V gene repertoire and very few N-region sequences in VDJ junctions. B-1 cells are the major producers of serum “natural antibodies”, which are typically low affinity, polyreactive, and IgM. Natural antibodies are present in individuals without overt immunization, and provide the first line of defense against influenza virus and encapsulated bacteria (Zhang and Carroll 2007).

The mature B cells (B-2) from the spleen can be divided into two main populations: the marginal zone (MZ) B cells and follicular (FO) B cells (Weill, Weller et al. 2009). MZ B cells are non-circulating mature B cells localizing in the marginal zone between the red pulp and the white pulp. They typically express high levels of IgM, CD21, CD1, CD9 with low levels of IgD, CD23, CD5, and CD11b that help to distinguish them from FO B cells and B-1 cells (Pillai, Cariappa et al. 2005). Similar to
B-1 cells, MZ B cells can be rapidly recruited into the early adaptive immune responses in a T cell-independent manner. The MZ B cells are especially well positioned as a first line of defense against systemic blood-borne antigens that enter the circulation and become trapped in the spleen. MZ B cells also display a lower activation threshold than their FO B cell counterparts with heightened propensity for plasma cell (PC) differentiation that contributes further to the accelerated primary antibody response (Pillai, Cariappa et al. 2005; Weill, Weller et al. 2009).

FO B cells are major constituents of B-2 cells and they reside in primary and secondary (germinal center-containing) lymphoid follicles. Naïve FO B cells display high levels of membrane IgM and IgD by alternative splicing, and their half-life is around 2 to 3 months; therefore called long-lived naïve mature cells (Martin and Kearney 2000). These cells recirculate in the blood, lymph, and lymphoid tissues. FO B cells are responsible for mounting a high affinity, long-lived antibody response, and also generate the memory response against a specific pathogen. When naïve FO B cells encounter antigen, they proliferate, upregulate activation markers such as CD80, CD86, and CD40, and process and present their antigen on the MHC II, priming them for T cell help (Natkunam 2007). When appropriate T cell help is supplied, the germinal center (GC) reaction begins. This process will be discussed in more detail below.

**Germinal center reaction and follicular helper T cells**

GCs are specialized structures within the secondary lymphoid tissues wherein antigen-specific B cells undergo clonal expansion, class switch recombination (CSR), and
somatic hypermutation (SHM). GC is associated with T-dependent antibody responses. During T-dependent antibody responses, naïve B cells originally present in the B-cell zone encounter Ags and migrate into the boundary between the T-cell zone and B-cell zone, wherein dendritic cell-activated T cells provide helper signals for the initial B cell activation. Then, some activated B cells migrate to the medullary cords of lymph nodes or splenic red pulps and differentiate into short-lived plasma cells. Other activated B cells move back to the B-cell zone to form GCs, wherein follicular helper T cells (T\textsubscript{FH}), which constitute approximately 5% to 20% of total GC cells, provide helper signals for B cell differentiation into long-lived, antibody-producing plasma cells and high-affinity memory B cells (Natkunam 2007).

As the GC matures, two distinct areas can be observed; the light zone (LZ), located distal to the T-cell zone, and dark zone (DZ), located adjacent to the T-cell zone. Rapidly proliferating germinal center B (GC-B) cells in the DZ, known as centroblasts, undergo SHM of their immunoglobulin (Ig) variable-region gene segments. Centrocytes, small GC-B cells present in the LZ, are considered to be a more differentiated form of centroblasts (Cannons, Qi et al. 2010). Since FDCs have the ability to capture large amounts of antigens in the form of immune complexes in highly ordered units termed iccosomes, centrocytes can bind antigens associated with FDCs using their newly expressed cell surface Ig, and they can process and present this Ag to T\textsubscript{FH} cells to receive helper signals. This interaction with T\textsubscript{FH} has several outcomes: 1) it results in the survival and selection of high-affinity centrocytes; 2) it provides signals for differentiation into long-lived plasma cells and high-affinity memory B cells; and 3) it can perpetuate GC
reactions by stimulating centrocytes to recycle to become centroblasts. In the absence of help from antigen-specific T_{H1}, centrocytes undergo apoptosis and removed by tingible body macrophages (Vinuesa, Tangye et al. 2005; King, Tangye et al. 2008).

**Plasmablasts and Memory B Cells**

Plasma cells are terminally differentiated, end-stage B cells that are dedicated to secrete large amounts of antibodies. They are responsible for the continuous maintenance of serum antibody levels (Hoyer, Manz et al. 2005; Dorner, Jacobi et al. 2009). After GC reactions, cells committed to plasma cells are referred to as plasmablasts (van Laar, Melchers et al. 2007). Plasmablasts are often identified by IgD^−, CD20^−, CD19^{dim}, CD38^+ phenotype, while plasma cells are identified as syndecan-1^+ (CD138^+) and HLA-DR^{low} cells (Calame, Lin et al. 2003). Whereas plasma cells do not divide, plasmablasts are still able to proliferate and are capable of temporarily producing antibodies in response to antigenic stimulation (Jourdan, Caraux et al. 2009). The expression of CXCR4 on the majority of plasmablasts is known to mediate localization of plasma cells in the bone marrow (by CXCL12), which provides niches for long-term survival of plasma cells. In contrast, CXCR3-expressing plasmablasts were shown to migrate into inflamed tissues (by CXCL9) (Odendahl, Mei et al. 2005).

Memory B cells are another main outcome after GC reaction, and are a central component of long-term humoral immunity. They retain high affinity B cell receptors which have undergone SHM and CSR. They are also able to effectively present antigen to T cells. In humans, memory B cells are defined as CD19^+ CD38^- population. Antigen-
specific memory B cells can be detected for over 50 years after vaccination (Fehniger, Cooper et al. 2003). They are known to self-renew by homeostatic proliferation (< 1 division per month), but not antigen-driven proliferation. Upon stimulation, memory B cells proliferate, and some replenish the memory cell pool and others differentiate into short- or long-lived plasma cells (Dorner, Jacobi et al. 2009).

**Signals for Plasma Cell Differentiation**

There are several cytokines or costimulatory molecules that promote the differentiation of B cells into antibody-producing plasma cells or plasmablasts, including CD40L, type I interferons (IFN-α and IFN-β), IL-2, IL-4, IL-6, IL-10, and IL-21 (Banchereau, Blanchard et al. 1993; Bryant, Ma et al. 2007). CD40L provided by activated T cells is critical for B cell help for the production of antibody (Banchereau 1995). Type I interferons and IL-6 are main cytokines produced by virus-infected plasmacytoid dendritic cells, and they were shown to act sequentially; type I interferons generate plasmablasts and IL-6 induce plasma cells from plasmablasts (Jego, Palucka et al. 2003). IL-10 mediates the differentiation of GC-B and memory B cells into plasma cells. Inability of IL-10 to differentiate naïve B cells can be overcome by addition of IL-2, resulting in the synergistic effect on antibody production. IL-4 was shown to direct GC-B cells into memory B cells with IgG4 or IgE isotype (Banchereau, Blanchard et al. 1993). IL-21 is the main cytokine produced by T_{FH} cells and the most potent cytokine driving all kinds of B cells into plasmablasts. B cell activating factor (BAFF) and CpG DNA (via
TLR9) were also reported to enhance plasma cell differentiation and antibody production (Bryant, Ma et al. 2007).

Five transcription factors play critical role in the transition of GC-B cells into plasmablasts or plasma cells. Pax-5 and Bcl-6 are important in maintaining GC-B cells. When plasma cell differentiation takes place, the master regulator Blimp-1 is first induced, which represses Bcl-6 and Pax5 expression while activating XBP-1. A recent study indicates that IRF-4 is also an important transcription factor, which upregulates Blimp-1 and other plasma cell specific genes (Johnson and Calame 2003; Calame 2006).

**Systemic Lupus Erythematosus (SLE)**

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmunedisease that affects millions of people worldwide. SLE is both gender and race specific. Whereas about 1 out of 2,000 people can develop SLE, about 1 out of 700 women and about 1 out of 245 African American women can develop this disease (Danchenko, Satia et al. 2006). It is generally accepted as a B cell-mediated autoimmune disease because of the production of pathogenic autoantibodies that affect multiple organs including skin, joints, and kidney. However, the involvement of autoreactive T cells in SLE pathogenesis is supported by T-cell depletion studies using lupus model mice (Steinberg, Roths et al. 1980; Wofsy, Ledbetter et al. 1985). The pathology of SLE can be manifested by a variety of symptoms such as skin rashes, arthritis, leukopenia, and proteinuria. Major autoantibodies found in SLE patients are anti-nuclear autoantibodies (ANA), which are found in 95% of SLE patients, anti-double stranded (ds) DNA, and anti-Smith (Sm)
antibodies. Currently, the exact etiology of SLE is unknown. However, it has been suggested that genetic factors predispose to SLE, but alone are not sufficient to trigger the disease; a gene-environment interaction is necessary (Cooper, Miller et al. 1999). Several hypotheses regarding the pathogenesis of SLE have been suggested. B cell intrinsic factors include aberrant B cell tolerance to self antigens, generating pathogenic autoantibodies. B cell extrinsic factors include defective clearance of apoptotic cells by phagocytes and loss of suppressive activity by regulatory immune cells (Gaipl, Munoz et al. 2007; Valencia, Yarboro et al. 2007; Dolff, Abdulahad et al. 2009; Dorner, Jacobi et al. 2009).

**Lupus Susceptibility Loci**

Large scale genome-wide linkage scans of families with multiple members affected with SLE have consistently demonstrated the presence of a susceptibility locus on human chromosome 1q23, which includes genes coding for SLAM family receptors (Tsao, Cantor et al. 1997; Moser, Gray-McGuire et al. 1999; Shai, Quismorio et al. 1999; Cantor, Yuan et al. 2004). Similarly, using lupus-prone mice such as MRL/lpr, NZB/NZW, and NZM2410, which spontaneously develop lupus, mouse chromosome 1 has also been shown to contain lupus susceptibility genes. In addition, the production of congenic mice derived from NZM2410 and C57BL/6 strains identified several murine lupus susceptibility loci; *Sle1* on chromosome 1, *Sle2* on chromosome 4, and *Sle3* on chromosome 7 (Morel, Yu et al. 1996). Fine mapping of the *Sle1* locus further identified three loci termed *Sle1a*, *Sle1b*, and *Sle1c*, which cause a loss of tolerance to chromatin
(Mohan, Alas et al. 1998). Sle1b, the most potent susceptibility locus on murine chromosome 1, harbors genes coding for SLAM family receptors, and susceptibility to lupus in B6.Sle1b congenic mice was shown to associate with extensive polymorphism in these SLAM family genes (Wandstrat, Nguyen et al. 2004).

**SLAM family receptors**

SLAM family receptors are widely expressed on hematopoietic cells, and play an important role in immune regulation. Members of this family are SLAM (SLAMF1, CD150), CD229 (SLAMF3, Ly-9), 2B4 (SLAMF4, CD244), CD84 (SLAMF5), NTB-A (SLAMF6; Ly108 in mouse), and CS1 (SLAMF7, CRACC, CD319). All SLAM family receptors except 2B4 are self-ligands, enabling homotypic cell-to-cell interactions. They have immunoreceptor tyrosine-based switch motifs (ITSMs) in their intracellular domain which can be bound by small adaptor proteins (SAP-family adaptors) such as SLAM-associated protein (SAP, \(SH2D1A\)), Ewing’s sarcoma (EWS)-activated transcript 2 (EAT-2, \(SH2D1B\)), and EAT-2-related transducer (ERT, \(SH2D1C\), only in rodents). Mutations in \(SH2D1A\), the gene encoding SAP, are responsible for a primary immunodeficiency termed X-linked lympho-proliferative disease (XLP) in humans (Nichols, Harkin et al. 1998). Studies using knockout mice deficient in SAP and SLAM family receptors indicate that SLAM family receptors and SAP play an important role in T cell-mediated help for humoral immunity (Wang, Satoskar et al. 2004; Howie, Laroux et al. 2005; Graham, Bell et al. 2006; Veillette, Zhang et al. 2008).
Recently, it was demonstrated that NK cells are able to perform immune surveillance against hematopoietic cells because of the restricted expression of SLAM family receptors in hematopoietic cells. Moreover, 2B4, CS1, and NTB-A expressed on NK cells not only play as an activating receptor when NK cells express abundant amount of SAP family adaptors, but also play as an inhibitory receptor when there is a relative paucity of SAP family adaptors (Dong, Cruz-Munoz et al. 2009).

Accumulating evidence supports the idea that SLAM family receptors are cell adhesion molecules as well as signaling molecules (Calpe, Wang et al. 2008; Schwartzberg, Mueller et al. 2009). For example, prolonged conjugate formation between T\textsubscript{FH} cells and GC-B cells requires homophillic interaction by CD84 for the optimal germinal center responses (Cannons, Qi et al. 2010). Another example includes the involvement of CD84 and SLAM in platelet aggregation (Nanda, Andre et al. 2005). However, the adhesive function of SLAM family receptors in other immune cells is currently unknown.

**2B4 (CD244)**

2B4 (CD244) is expressed on NK cells, some CD8\textsuperscript{+} T cells, monocytes, basophils, and eosinophils. In both mice and humans, CD48 is the ligand for 2B4 (Brown, Boles et al. 1998; Latchman, McKay et al. 1998). Our laboratory originally identified, cloned and characterized the 2B4 receptor in the mouse (Garni-Wagner, Purohit et al. 1993; Mathew, Garni-Wagner et al. 1993). In the mouse, two isoforms of 2B4, m2B4-L and m2B4-S, are expressed which are the products of differential splicing of hnRNA (Stepp, Schatzle et al. 1998).
1999). These two isoforms differ only in the cytoplasmic domain, and they send opposing signals to NK cells (Schatzle, Sheu et al. 1999). Human NK cells also express two isoforms of 2B4, h2B4-A and h2B4-B, which differ in a small portion of the extracellular domains (Kumaresan and Mathew 2000; Mathew, Rao et al. 2009).

The important role of 2B4 has been implicated in various infection and clinical settings. Human 2B4 expression is upregulated on T cells in response to viral infections including HIV (human immunodeficiency virus), HSV (herpes simplex virus), and CMV (cytomegalovirus) (Garland, El-Shanti et al. 2002). The expression of CD48 on B cells is upregulated by EBV (Epstein-Barr virus). Soluble CD48 (ligand for 2B4) is also detected at elevated levels in the plasma of patients with arthritis and lymphoid leukemia (Smith, Biggs et al. 1997). It has been shown that 2B4-CD48 interactions provide a costimulatory signal among T cells and regulate the proliferation of activated/memory T cells (Kambayashi, Assarsson et al. 2001; Lee, Bhawan et al. 2003). Furthermore, ligation of CD48 delivers an accessory signal for CD40-mediated activation of human B cells (Klyushnenkova, Li et al. 1996).

2B4 is the most well characterized receptor among SLAM family members. Engagement of 2B4 by its ligand CD48 results in phosphorylation of 2B4 followed by the association of SAP. SAP-dependent 2B4 signaling pathway seems to involve PI3K pathway, which is linked to IFN-γ production, whereas LAT-PLC-γ pathway are linked to 2B4-mediated NK cell killing (Aoukaty and Tan 2002; Tassi and Colonna 2005). Recently, it was demonstrated that 2B4 may function as activating receptor as well as inhibitory receptor both in human and murine NK cells (Chlewicki, Velikovsky et al. 2005).
Three factors can dictate the result after engagement of 2B4. First, the abundance of 2B4 in NK cells prevents killing activity by NK cells. Second, the abundance of CD48, therefore the abundance of 2B4 crosslinking, inhibits cytotoxic function of NK cells. Third, the relative scarcity of SAP blocks NK-mediated cellular cytotoxicity. Since NK cells express 2B4 as well as CD48, enough 2B4-CD48 interactions prevent NK cell fratricide (Taniguchi, Guzior et al. 2007). Recently, 2B4 was shown to associate with MHC class I molecule, thereby restricting 2B4-mediated self-killing of NK cells (Betser-Cohen, Mizrahi et al. 2010).

Also, 2B4 is the only receptor whose promoter has been well studied among SLAM family members. The 2B4 promoter has several AP-1 binding sites at -348 region and -106 to -100 region, acting as proximal promoter (Chuang, Pham et al. 2001). In addition, -1151 to -704 region of 2B4 promoter has Ets element which act as positive regulatory region (Vaidya and Mathew 2005). -653 to -540 region was responsible for inhibiting transcriptional activity of 2B4. Interestingly, 2B4 engagement by its ligand CD48 or agonist anti-2B4 antibody C1.7 resulted in temporal reduction of surface expression of 2B4 due to receptor endocytosis as well as reduced transcriptional activity involving Ets-1 element (Sandusky, Messmer et al. 2006; Mathew, Vaidya et al. 2007).

**CS1 (CRACC or CD319)**

CS1, also called CRACC or CD319, was first identified by our laboratory and Dr. Colonna’s laboratory in 2001 (Boles and Mathew 2001; Bouchon, Cella et al. 2001). CS1 is expressed on NK cells, activated T cells, activated B cells and mature dendritic cells.
CS1 is a self-ligand, thereby enabling homotypic interactions between CS1-expressing cells (Kumaresan, Lai et al. 2002). Two isoforms of CS1, CS1-L and CS1-S are expressed in NK cells. These two isoforms differ in their cytoplasmic domain and signal differently (Lee, Boles et al. 2004). Unlike other SLAM members, CS1 transmits SAP-independent but EAT-2-dependent signaling in human NK cells, resulting in cytolytic activity toward tumor target cells (Bouchon, Cella et al. 2001; Tassi and Colonna 2005). Recent CS1-knock out mice study revealed that CS1 can play both activating and inhibitory functions, depending on EAT-2 expression (Cruz-Munoz, Dong et al. 2009). Signaling Molecules activated by CS1 engagement include PLC\(\gamma\), ERK1/2, PI3K, AKT and STAT3 (Tassi and Colonna 2005; Tai, Soydan et al. 2009).

In human B lymphocytes, CS1 expression is ignorable in resting peripheral B cells (Bouchon, Cella et al. 2001). However, the expression of CS1 can be induced after PMA treatment or CD40-mediated B cell activation (Bouchon, Cella et al. 2001; Murphy, Hobby et al. 2002; Lee, Mathew et al. 2007). Our laboratory previously demonstrated that CS1 induces B cell proliferation and leads to the production of autocrine cytokines such as IL-14 (Lee, Mathew et al. 2007), which plays a role in the development of autoimmunity (Ambrus, Contractor et al. 1995; Shen, Zhang et al. 2006; Shen, Suresh et al. 2009). Another report showed that CS1 is involved in homotypic B cell adhesion (Murphy, Hobby et al. 2002). Recently, it has been reported that CS1 is overexpressed in multiple myeloma (MM) and an anti-CS1 humanized monoclonal antibody (HuLuc53, elotuzumab) inhibited myeloma cell adhesion and induced antibody mediated cellular cytotoxicity in bone marrow milieu (Hsi, Steinle et al. 2008; Tai, Dillon et al. 2008). CS1
promotes multiple myeloma cell adhesion, clonogenic growth, and tumorigenicity via cmaf-mediated interactions with bone marrow stromal cells (Tai, Soydan et al. 2009). Currently, elotuzumab is in human clinical trial for MM.

Objectives

2B4 and CS1 belong to the recently recognized SLAM family of receptors, and they play important roles in immune regulation. In particular, recent SLE family-based association studies suggest a possible role of mutations in 2B4 and CS1 in SLE disease. However there have been no studies on the expression of these receptors in human clinical patients with SLE. Chapter 2 will reveal whether SLE patients display altered expression of 2B4 and CS1 in their immune cells. Chapter 3 will identify a distinct SLE B cell population, whose increase was shown to associate with disease severity. Chapter 4 will describe how CS1 gene expression is transcriptionally regulated. Overall, these studies will provide important information about pathogenesis of SLE. Ultimately, these information can help developing new therapeutics against SLE and other B-cell mediated diseases.
CHAPTER II

EXPRESSION OF 2B4 AND CS1 IN SLE

SUMMARY

Genome-wide linkage analyses of human chromosomes and characterization of murine lupus susceptible genes have shown a strong association of SLE with SLAM family receptors. However, there have been no studies on the expression of SLAM family receptors in patients with SLE. 2B4 (CD244) and CS1 (CD319) are two members of the SLAM family receptors that regulate NK, T, and B cell functions. We hypothesize that 2B4 and CS1 may mediate the immune dysregulation observed in patients with SLE. The purpose of this study was to investigate the expression of 2B4 and CS1 in peripheral blood mononuclear cells (PBMCs) from patients with SLE. The mRNA expression of splice variants of 2B4 and CS1 in total PBMCs was analyzed by RT-PCR. The surface expression of CS1 and 2B4 on total PBMCs, T, B, NK cells, or monocytes was analyzed by flow cytometry. Altered mRNA expression ratio between splice variants was more notable in 2B4 than in CS1, although it did not reach statistical significance. 2B4 was downregulated in SLE as shown by reduced mean fluorescence intensity ratio (MFIR) in all 2B4-expressing cells as well as decreased proportion of 2B4-positive cells in NK cells and monocytes. Most strikingly, increased proportion of CS1-positive B cells was observed in patients with SLE compared to healthy individuals. In conclusion, this study demonstrates that the expression of 2B4 and CS1 is altered in patients with SLE.
INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease, characterized by the improper regulation of B cells that leads to the production of auto-antibodies. The incidence of disease is gender-biased, with a female to male ratio of 9:1, and the onset of disease is usually during the child-bearing years (Wakeland, Liu et al. 2001). Using lupus-prone mice such as MRL/lpr, NZB/NZW, and NZM2410, which spontaneously develop lupus, mouse chromosome 1 has been shown to contain lupus susceptibility genes (Kono, Burlingame et al. 1994; Morel, Rudofsky et al. 1994; Drake, Rozzo et al. 1995; Morel, Yu et al. 1996). Genomic characterization of the Sle1b locus, the most potent member of lupus susceptibility region on murine chromosome 1, identified a highly polymorphic cluster of genes coding for the SLAM family receptors (Wandstrat, Nguyen et al. 2004). Similarly, genome-wide linkage analyses of SLE families have shown strong association of SLE with the 1q23 region of human genome, which also includes SLAM family receptors (Tsao, Cantor et al. 1997; Moser, Neas et al. 1998; Tsao, Cantor et al. 2002).

SLAM family receptors are broadly expressed on hematopoietic cells, and play an important role in immune regulation. Members of this family are SLAM (SLAMF1, CD150), CD229 (SLAMF3, Ly-9), 2B4 (SLAMF4, CD244), CD84 (SLAMF5), NTB-A (SLAMF6; Ly108 in mouse), and CS1 (SLAMF7, CRACC, CD319). All these receptors have immunoreceptor tyrosine-based switch motifs (ITSMs) in their intracellular domain which can be bound by small adaptor proteins such as SLAM-associated protein (SAP,
SH2D1A), Ewing’s sarcoma (EWS)-activated transcript 2 (EAT-2, SH2D1B), and EAT-2-related transducer (ERT, SH2D1C, only in rodents). Mutations in SH2D1A, the gene encoding SAP, are responsible for a primary immunodeficiency X-linked lymphoproliferative disease (XLP) in humans (Nichols, Harkin et al. 1998). Studies using knockout mice deficient in SAP and SLAM family receptors indicate that SLAM family receptors and SAP play an important role in T cell-mediated help for humoral immunity (Wang, Satoskar et al. 2004; Howie, Laroux et al. 2005; Graham, Bell et al. 2006; Veillette, Zhang et al. 2008).

A comprehensive haplotype-based study using 630 kb region of SLE susceptibility locus on human chromosome 1q23 has identified 143 single nucleotide polymorphisms (SNPs) and CS1 gene had the highest hit (Farwell 2004). Human CS1 gene was originally identified and cloned by our laboratory (Boles and Mathew 2001). CS1 is expressed on NK cells, activated T cells, activated B cells and mature dendritic cells (Bouchon, Cella et al. 2001). CS1 is a self-ligand and homophilic interaction of CS1 activates NK cell cytolytic function (Kumaresan, Lai et al. 2002). CS1 recruits EAT-2 as an adaptor molecule and exerts activating or inhibitory functions depending on EAT-2 expression (Tassi and Colonna 2005; Cruz-Munoz, Dong et al. 2009). Recently, it has been reported that CS1 is expressed at high levels in normal plasma cells and multiple myeloma cells, and serum levels of circulating CS1 correlate with active multiple myeloma (Hsi, Steinle et al. 2008; Tai, Dillon et al. 2008; Tai, Soydan et al. 2009). Importantly, we found that CS1 induces proliferation and production of autocrine cytokines in B lymphocytes (Lee, Mathew et al. 2007).
Also, a large scale, case-control association study suggested that polymorphisms in 2B4 (CD244) increase the risk for developing SLE as well as rheumatoid arthritis (Suzuki, Yamada et al. 2008). 2B4 is expressed on NK cells, some CD8+ T cells, monocytes, basophils, and eosinophils. 2B4 acts as an activating receptor or inhibitory receptor depending on the availability of adaptor protein SAP (Chlewicki, Velikovsky et al. 2008). In both mice and humans, the ligand of 2B4 is CD48, which is expressed by all hematopoietic cells (Brown, Boles et al. 1998; Latchman, McKay et al. 1998). The important role of 2B4-CD48 interaction has been implicated in various infection and clinical settings. Human 2B4 expression is upregulated on T cells in response to viral infections including HIV (human immunodeficiency virus), HSV (herpes simplex virus), and CMV (cytomegalovirus) (Peritt, Sesok-Pizzini et al. 1999; Garland, El-Shanti et al. 2002; Zaunders, Dyer et al. 2004). The expression of CD48 on B cells is upregulated by EBV (Epstein-Barr virus) infection (Yokoyama, Staunton et al. 1991; Klaman and Thorley-Lawson 1995). Soluble CD48 is also detected at elevated levels in the plasma of patients with arthritis and lymphoid leukemia (Smith, Biggs et al. 1997). It was shown that 2B4-CD48 interactions provide a costimulatory signal among T cells and regulate the proliferation of activated/memory T cells (Kambayashi, Assarsson et al. 2001; Lee, Bhawan et al. 2003). Furthermore, ligation of CD48 delivers an accessory signals for CD40-mediated activation of human B cells (Klyushnenkova, Li et al. 1996).

Previously, Kumar et al. showed that altered expression of splice variants of Ly108, the murine homolog of NTB-A, can lead to autoimmunity (Kumar, Li et al. 2006). Whereas Ly108.2 contains 3 ITSMs in the intracellular domain, Ly108.1 contains only 2
ITSMs (Zhong and Veillette 2008). These two isoforms were most differentially expressed on immature B cells between wild-type and lupus-prone mice. Whereas the normal Ly108.2 isoform sensitized immature B cells to transmit B cell receptor (BCR) signals for self-tolerance such as clonal deletion and BCR editing, the lupus-associated Ly108.1 failed to transmit such signals in immature B cells. Our laboratory previously identified two splice variants of CS1, CS1-L and CS1-S (Lee, Boles et al. 2004). The CS1 gene contains seven exons, and the last three exons encode the cytoplasmic domain. CS1-S form is generated due to the elimination of exon 5, which leads to a frameshift immediately after the transmembrane region causing early termination of translation, thus resulting in a 296-amino-acid-long polypeptide rather than a 335-amino-acid-long polypeptide. Since CS1-S does not have C-terminal 39 amino acids, it does not transmit signals. Interestingly, we found that human B lymphocytes do not express CS1-S isoform (Lee, Mathew et al. 2007). Also, human 2B4 is alternatively spliced to generate h2B4-A and h2B4-B (Kumaresan and Mathew 2000). Human 2B4 gene is composed of nine exons, and the splice acceptor near 5’ region of exon 3 in h2B4-B is located in 15 nucleotides upstream of 5’ end of exon 3 in h2B4-A. Therefore, h2B4-B has additional five amino acids between V and C2 domains in the extracellular region, which is important for the ligand binding. We previously found that IL-2-activated NK cells only express 2B4-A (Mathew, Rao et al. 2009).

Based on the high polymorphisms found in 2B4 and CS1, the strong association of these genes with SLE, and the existence of splice variants, we hypothesized that the alterations in the expression of SLAM family receptors 2B4 and CS1 may mediate the
immune dysregulation observed in patients with SLE. The focus of this study is to compare the expression of 2B4 and CS1 in SLE patients versus those of healthy controls. Most strikingly, increased proportion of CS1-positive B cells was observed in patients with SLE compared to healthy individuals. In addition, 2B4 was downregulated in SLE patients as shown by reduced mean fluorescence intensity ratio (MFIR) in all 2B4-expressing cells as well as decreased proportion of 2B4-positive cells in NK cells and monocytes. Altered mRNA expression ratio between splice variants was more notable in 2B4 than in CS1, although it did not reach the statistical significance. In conclusion, this study demonstrated that the expression of 2B4 and CS1 is altered in patients with SLE. A possible contribution of this altered expression of 2B4 and CS1 will be discussed
MATERIALS AND METHODS

Patients and healthy control volunteers

Blood samples were obtained from 45 patients diagnosed with SLE (two males, 43 females) at John Peter Smith (JPS) Hospital, Fort Worth, TX and from 30 healthy volunteers at University of North Texas Health Science Center (UNTHSC), Fort Worth, TX with prior approval from Internal Review Board of JPS Health Network and UNTHSC. Written informed consents were obtained from all of the study subjects. Patients with SLE were classified according to the 1997 revised criteria by the American College of Rheumatology (Tan, Cohen et al. 1982; Hochberg 1997). General characteristics of SLE patients in our study were summarized in TABLE 2.1. Eight patients had active SLE, defined by SLEDAI score of ≥8 (Bombardier, Gladman et al. 1992). All 45 patients were positive for anti-nuclear antibody (ANA). Also, clinical and demographical characteristics for each individual patient, including SLE Disease Activity Index (SLEDAI), treatments, major disease manifestations and serological parameters, are shown in TABLE 2.2.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from ethylene-diamine-tetra-acetic acid (EDTA)-treated whole-blood samples by Histopaque-1077 (Sigma Chemicals, St. Louis, MO) density gradient centrifugation using LeucoSep tubes
(Greiner, Monroe, NC). The remaining red blood cells were lysed with ACK lysis buffer. Resulting PBMCs were used for immunostaining or RT-PCR.

**Antibodies and immunostaining for flow cytometry analysis**

Before starting immunostaining, PBMCs were incubated with human IgG Fc fragments (Rockland, PA) for prevention of possible Fc receptor-mediated fluorescence. Tricolor staining (FITC-PE-APC) method was applied for immunostaining. Anti-hCD3-FITC (clone UCHT1; BD Biosciences, San Jose, CA) / anti-hCD19-APC (clone J4.119, Beckman Coulter, Miami, FL) or anti-hCD14-FITC (clone M5E2, BD Biosciences) / anti-hCD56-APC (clone N901 (NKH-1), Beckman Coulter) were used in combination with anti-h2B4-PE (clone C1.7, Beckman Coulter) or anti-hCS1-PE (clone 235614, R&D Systems, Minneapolis, MN). For isotype controls, mouse IgG1-FITC, mouse IgG1-PE, mouse IgG2a-PE, and mouse IgG1-APC were used (all from Caltag Laboratories, Burlingame, CA). Samples were run on a Cytomics FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA). Data were analyzed using CXP software (Beckman Coulter). Mean fluorescence intensity ratio (MFIR) was calculated by dividing the mean fluorescence intensity of samples with the mean fluorescence intensity of isotype controls.

**Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and gel electrophoresis for splice variants of 2B4 and CS1**

Some PBMCs were dissolved with RNA STAT-60 in 5 million cells per 1 ml and kept at -80°C until RNA extraction. RNA was extracted by chloroform and precipitated
by isopropanol. After resuspension with 0.1% diethylpyrocarbonate (DEPC)-water, RNA purity and concentration were determined by measuring optical density at 260, 280 and 230nm. 2 µg of RNA was used for cDNA synthesis in the presence of primer mixture of random hexamer (New England Biolabs, Ipswich, MA) and oligo dT (Integrated DNA Technologies, Coralville, IA). After RT reaction, cDNA was diluted in the concentration of 100ng/µl and 1 to 3 µl was used for each PCR reaction as a template. We used PCR primers simultaneously amplifying two splice variants of CS1 and 2B4. CS1-F727 primer (5’-GTC TCT TTG TAC TGG GGC TAT TTC-3’) recognizes exon 4 of CS1 gene and CS1-R955 primer (5’-TTT CCA TCT TTT TCG GTA TTT-3’) recognizes exon 6 and 7 of CS1 gene, therefore generating 228bps and 125bps of CS1-L and CS1-S PCR products, respectively. 1H2B4-FP primer (5’-CCT CTA CTG CCT GGA GGTCAC CAG-3’) binds to exon 2 of 2B4 gene and 1H2B4-RP primer (5’-CCA CTT GGC ATC TCC CTC TGT CC-3) binds to exon 3 of 2B4 gene, resulting in 137bps and 152bps of 2B4-A and 2B4-B PCR products. GAPDH primers (FP, 5’-ATG ACA TCA AGA AGG TGG TG-3’; RP, 5’-CAT ACC AGG AAA TGA GCT TG-3’) were used for internal control. PCR cycle conditions were 94°C for 45s, 50°C for 45s and 72°C for 60s, repeated for 35 cycles using Taq DNA polymerase (New England Biolabs, Ipswich, MA). CS1 PCR products were run on 2% agarose gels. 2B4 PCR products were electrophoresed on 8 to 12% non-denaturing polyacrylamide gels. Intensity of PCR bands was estimated using the Area Density Tool of LabWorks software (UVP, Upland, CA).

Statistical analysis
Two-tailed student t-test was performed to determine significant differences between the SLE patients and healthy individuals. If variances were significantly different between the two populations, Welch’s correction was applied to calculate the p value. P values below 0.05 were considered statistically significant. Data were analysed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA).
RESULTS

The mRNA expression of splice variants of CS1 and 2B4 in SLE

A previous study showing that differential expression ratio of splice isoforms in Ly108 can cause the development of lupus in mice, raises a possibility that altered expression of splice variants of SLAM family receptors may transmit differential signals because of the differences in the number of ITSMs in their intracellular domains (Kumar, Li et al. 2006). Interestingly, similar to Ly108, we previously found that CS1-L that contains two ITSMs functions as an activating receptor while CS1-S that has no ITSMs does not display any signaling function in NK cells (FIGURE 2.1)(Lee, Boles et al. 2004). Therefore, we were interested in determining whether altered expression of splice variants of CS1 can be observed in human SLE.

In order to do this, we determined the expression ratio of CS1-L over CS1-S mRNA in total PBMCs from patients with SLE and healthy individuals by RT-PCR. Common PCR primers detecting both CS1 isoforms generated PCR products of 228 bps for CS1-L and 125 bps for CS1-S (TABLE 2.2 & FIGURE 2.2). As seen in FIGURE 2.3, all healthy individuals and most of SLE patients expressed three- to six-fold higher levels of CS1-L than CS1-S. However, some SLE patients expressed higher levels of CS1-S isoform, resulting in lower values of CS1-L/S ratio (SLE 19, SLEDAI=4 and SLE 36, SLEDAI=0). Notably, one patient showed no expression of CS1-S form (SLE 17;
SLEDAI=4). However, there was no significant difference in the expression ratio of CS1-L over CS1-S mRNA between healthy controls and SLE patients (FIGURE 2.4).

A recent family-based association study in a Japanese population identified 5 SNPs in the introns of 2B4 associated with SLE: rs6682654 (intron 3), rs1319651 (intron 4), rs3766379 (intron 5), rs3753389 (intron 5), and rs11265493 (intron 7) (Suzuki, Yamada et al. 2008). Since mutations in the intron sequence can affect splicing events, we decided to see whether differential expression of splice variants of human 2B4 is observed in SLE patients. Our laboratory has originally identified two different splice variants of h2B4, h2B4-A and h2B4-B (Kumaresan and Mathew 2000). While h2B4-A and h2B4-B share the same intracellular domains, h2B4-B has additional 5 amino acids between V and C2 region compared to h2B4-A (FIGURE 2.5). Recently, we have shown that only 2B4-A can interact with its ligand CD48 (Mathew, Rao et al. 2009).

In order to examine whether splice isoforms of h2B4 are differentially expressed in SLE, we analyzed mRNA expression of h2B4-A and h2B4-B in total PBMC from patients with SLE and healthy control by RT-PCR. We used common PCR primers detecting both h2B4-A and h2B4-B forms, which generates PCR products of 137 bps for h2B4-A and 152 bps for h2B4-B (TABLE 2.2, FIGURE 2.6). Because of the small difference in size between h2B4-A and h2B4-B, the PCR products were electrophoresed on 8 to 12 % non-denaturing polyacrylamide gels. As seen in FIGURE 2.7, healthy individuals expressed five- to eight-fold higher levels of 2B4-A than 2B4-B. However, some patients with SLE showed more predominance (more than 10-fold) of h2B4-A over h2B4-B than in healthy controls (SLE 16, SLEDAI=4; SLE22, SLEDAI=4; and SLE27,
SLEDAI=2). Interestingly, some patients with active SLE showed comparable level of 2B4-A and 2B4-B (SLE1, SLEDAI=15; SLE3, SLEDAI=12; and SLE4, SLEDAI=10). Although these data suggest that splicing of 2B4 mRNAs might be differentially regulated during the pathogenesis of SLE, the comparison of mRNA expression ratio of h2B4-A over h2B4-B between SLE patients and healthy individuals did not reach the statistical significance (FIGURE 2.8).

**Analysis of the surface expression of 2B4 in SLE**

It is generally accepted that lymphopenia in SLE patients occurs during acute phases of the disease. In order to find whether our SLE patients also display similar features, we analyzed the proportion of CD3\(^+\) T cells, CD19\(^+\) B cells, CD56\(^+\) NK cells and CD14\(^+\) monocytes in the total PBMCs from healthy controls vs. patients with SLE by flow cytometry. As seen in TABLE 2.3, our SLE patients showed decreased proportion of CD3\(^+\) T cells and CD56\(^+\) NK cells in PBMCs. However, there was a relative increase in the proportion of monocytes in SLE patients (TABLE 2.3).

Next, we examined the proportion of 2B4-expressing cells in total PBMCs, CD3\(^+\) T cells, CD56\(^+\) NK cells, and CD14\(^+\) monocytes in patients with SLE and healthy controls. As shown in FIGURE 2.9, the proportion of 2B4-positive cells in total PBMCs and T cells was not significantly different between healthy controls and SLE patients (Panel A and B). However, the proportion of 2B4-expressing cells was significantly decreased in CD56\(^+\) NK cells and CD14\(^+\) monocytes from patients with SLE compared to healthy controls (Panel C and D). Although all monocytes are known to express 2B4,
monocytes from two patients with SLE (SLE7, SLEDAI=8 and SLE17, SLEDAI=4) showed almost no expression of 2B4.

Since the proportion of cells can be affected by a relative lymphopenia in patients with SLE, we also determined the mean fluorescence intensity ratio (MFIR), which represents the density of surface receptors at the single-cell level. MFIR value was obtained by gating only 2B4-expressing cells in total PBMCs, CD3+ T cells, CD56+ NK cells, and CD14+ monocytes. Interestingly, when we compared MFIR for 2B4 expression between SLE patients and healthy controls, MFIR was significantly lower in SLE compared to healthy controls. This suggests that the surface expression of 2B4 was downregulated by all 2B4-expressing cells including total PBMCs, CD3+ T cells, CD56+ NK cells, and CD14+ monocytes (FIGURE 2.10).

Collectively, altered mRNA expression of 2B4 splice variants, decreased proportion of 2B4+ NK cells and 2B4+ monocytes, and reduced surface expression of 2B4 in all 2B4-expressing cells in SLE suggests that altered expression of 2B4 may play a role in SLE pathogenesis.

Analysis of the surface expression of CS1 in SLE

In order to determine whether the surface expression of CS1 is also altered in SLE, flow cytometric analysis was performed to find the proportion of CS1-expressing cells in total PBMCs, CD3+ T cells, CD19+ B cells, and CD56+ NK cells in patients with SLE and healthy individuals. As shown by FIGURE 2.11, the proportion of CS1-expressing cells in total PBMCs, T cells, and NK cells was not significantly different between
healthy controls and patients with SLE (Panel A, B, and C). However, SLE B cells contained significantly higher proportion of CS1-positive cells than healthy control B cells did (Panel D). Since the proportion of B cells are not different between SLE patients and healthy individuals (TABLE 2.3), increased proportion of CS1+ B cells may be interpreted as increased number of CS1+ B cells in SLE.

Again, the density of surface CS1 was determined by measuring MFIR. As seen in FIGURE 2.12, MFIR of CS1+ cells in total PBMCs was not significantly different between healthy controls and SLE patients. However, the surface expression of CS1 was significantly upregulated by CS1+ T cells, whereas it was significantly downregulated by CS1+ NK cells in SLE. For analysis of B cells, we gated total B cells including both CS1+ and CS1- B cells, because percentage of CS1+ B cells were very low in healthy controls. Despite the significant percent increase of CS1+ B cells, MFIR for CS1 expression within total B cells was not significantly different between SLE patients and healthy controls.

These data suggests that the expression of CS1 is dynamically regulated by subsets of lymphocytes in SLE. Considering that B cells are the producers of pathogenic autoantibodies, the most significant finding of this chapter may be the increased proportion of CS1-expressing B cells in SLE.
DISCUSSION

This study for the first time showed that the expression of 2B4 AND CS1 is altered in human SLE patients. The expression ratio of alternative splice transcripts of 2B4 and CS1 in total PBMCs was altered in some patients with SLE. However, there was no statistical difference of expression ratio of splice variants of 2B4 and CS1 between healthy controls and SLE patients. Alterations of expression ratio of 2B4 and CS1 splice variants may reach statistical significance if sorted cells are used for analysis. In addition, there was a decreased proportion of 2B4-expressing NK cells and monocytes in SLE. Furthermore, reduced surface 2B4 expression is found in total PBMCs, including T cells, NK cells, and monocytes in SLE. Since 2B4-CD48 interaction plays an important role in NK cell cytotoxicity against hematopoietic cells, reduced 2B4 expression may indicate a possible role of NK cells in SLE pathogenesis. Moreover, CS1 was shown to be overexpressed in T cells and downexpressed in NK cells in SLE. Most strikingly, CS1-expressing B cells were significantly increased in SLE patients compared to healthy volunteers. As SLE is a B cell-mediated autoimmune disease, CS1-expressing SLE B cells were further investigated in next chapter.
TABLE 2.1. Summary of characteristics of patients with SLE.

<table>
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<th>Value</th>
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</tr>
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</tr>
<tr>
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<td>2 (4)</td>
</tr>
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<td>Hispanic</td>
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</tr>
<tr>
<td>Asian American</td>
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</tr>
<tr>
<td>Age, mean ± SD years</td>
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</tr>
<tr>
<td>Duration of SLE, mean ± SD years</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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</tr>
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<td>Anti-Sm positive</td>
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<tr>
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<tr>
<td>Inactive SLE (SLEDAI &lt;8)</td>
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<tr>
<td>Proteinuria</td>
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<td>Leukopenia</td>
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<td>Methotrexate</td>
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<tr>
<td>Others</td>
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</table>

* Except where indicated otherwise, values are the number (%) of the patients. ANA = anti-nuclear antibody; Anti-dsDNA = anti-double-stranded DNA; Anti-Sm = anti-smith antigen; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index.
### TABLE 2.2. Clinical and demographic characteristics of patients with SLE

<table>
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<tr>
<th>Patient</th>
<th>Race</th>
<th>Age/sex</th>
<th>Disease Duration (months)</th>
<th>Family History</th>
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<th>Anti-dsDNA</th>
<th>Anti-Sm</th>
<th>SLEDAI</th>
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<td>+</td>
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<td>12</td>
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<td>15</td>
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<td>-</td>
<td>-</td>
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<td>12</td>
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ANA = anti-nuclear antibody; Anti-dsDNA = anti-double-stranded DNA; Anti-Sm = anti-Smith antigen; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; AA = African American; H = Hispanic; A = Asian; C = Caucasian; F = female; M = male; ND = not determined; R = rash; HL = hair loss; AR = arthritis; LP = leukopenia; PU = proteinuria; LCL = low complement levels; IDB = increased DNA binding; Pred (X) = prednisone (mg/day); HCQ = hydroxychloroquine; AZA = azathioprine; MPA = mycophenolic acid; MTX = methotrexate.
TABLE 2.3 Nucleotide sequences used in PCR amplification of splice variants

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<th>Name</th>
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<th>Position*</th>
<th>Expected size</th>
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<td>2B4-A: 488-511</td>
<td>2B4-A: 137bp</td>
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<td></td>
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* Positions are according to GenBank accession number AF261085, AF117711, AF242540, AF291815, and BC027867 for GAPDH, 2B4-A, 2B4-B, CS1-L and CS1-S, respectively.
FIGURE 2.1 Schematic representations of CS1-L and CS1-S. V represents Ig-V-like domain, C2 represents Ig-C2-like domain, Y1 and Y2 represent immunoreceptor tyrosine-based switch motif (TxYxxV/I).
FIGURE 2.2 Schematic of the CS1 gene structure and alternative splicing. CS1 gene contains seven exons. Exon 1 corresponds to 5’ untranslated region (UTR) and signal sequence (SS). Exon 2 encodes the V domain. Exon 3 encodes the C2 domain. Exon 4 encodes the transmembrane (TM) domain. Exon 5, 6 and 7 encodes the cytoplasmic (Cyt) domains. CS1-S form is generated due to the absence of exon 5, which results in a frameshift and early termination of translation. Whereas CS1-L has two tyrosine-containing motifs (Y1 and Y2), CS1-S has none.
FIGURE 2.3 Altered expression ratio of splice isoforms of human CS1 in SLE. Differential expression of two CS1 transcript variants, CS1-L and CS1-S, was analysed by RT–PCR using PBMCs from SLE patients and healthy controls. PCR products were run on 2% agarose gels. Lanes 1 and 2 are positive controls amplified from plasmid clones for CS1-L and CS1-S, respectively. Low values of a CS1 L/S ratio were found in some SLE patients (patient 19, SLEDAI= 4 and patient 36, SLEDAI = 0). Notably, one patient showed no expression of CS1-S isoform (patient 17; SLEDAI = 4).
FIGURE 2.4 Comparison of the ratio of CS1-L and CS1-S splice isoforms in total PBMCs between SLE patients (SLE) (n=30) and healthy controls (HC) (n=27).
FIGURE 2.5 Schematic representations of 2B4-A and 2B4-B. V represents Ig-V-like domain, C2 represents Ig-C2-like domain, Y1 through Y4 represent immunoreceptor tyrosine-based switch motif (TxYxxV/I). Additional five amino acids are between V and C2 domain.
FIGURE 2.6 Schematic of the 2B4 gene structure and alternative splicing. Human 2B4 gene is composed of nine exons. Exon 1 corresponds to the 5’ untranslated region (UTR) and signal sequence (SS). Exon 2 encodes the V domain. Exon 3 codes for the C2 domain. Exon 4 encodes the transmembrane (TM) domain and the first 10 amino acids of the cytoplasmic (Cyt) domain. The cytoplasmic domain is encoded by exon 5-8 and part of exon 9. 5’ region of exon 3 has two splice acceptor sites. The splice acceptor site used by 2B4-B is 15 nucleotides upstream of the one used by 2B4-A. Therefore, 2B4-B has additional five amino acids between V and C2 domains in the extracellular region.
FIGURE 2.7 Altered expression ratio of spliced isoforms of human 2B4 in SLE. Differential expression of two human 2B4 transcript variants, 2B4-A and 2B4-B, in SLE was analysed by semiquantitative RT-PCR. PCR products were electrophoresed on 8 to 12% non-reducing polyacrylamide gels. Lane 1, 2, and 3 are positive controls amplified using h2B4-A plasmid clone, h2B4-B plasmid clone, and 1:1 mixture of h2B4-A and -B as templates, respectively. While some SLE patients (Patient 16, SLEDAI=4, Patient 22, SLEDAI=4, and Patient 27, SLEDAI=2) have dominant expression of h2B4-A, patients with active SLE (Patient 1, SLEDAI=15, Patient 3, SLEDAI=12, and Patient 4, SLEDAI=10) have comparable level of expression in h2B4-A and h2B4-B.
<table>
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<th>2B4-B</th>
<th>2B4-A&amp;B</th>
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<th>HC3</th>
<th>HC4</th>
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<th>SLE42</th>
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<th>SLE16</th>
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<th>SLE3</th>
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<tr>
<td>2B4-A/2B4-B ratio</td>
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<td>7.4</td>
<td>5.2</td>
<td>5.3</td>
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</table>
FIGURE 2.8 Comparison of the ratio of 2B4-A and 2B4-B splice isoforms in total PBMCs between SLE patients (SLE) (n=30) and healthy controls (HC) (n=27).
### TABLE 2.4 Percentage of immune cells in PBMCs from patients with SLE vs. healthy donors

<table>
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<tr>
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<th>SLE Patients (N=45)</th>
<th>P level</th>
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<td>CD3⁺ T cells</td>
<td>58.50 ± 11.30</td>
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<tr>
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<tr>
<td>CD14⁺ monocytes</td>
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<td>2B4⁺ cells</td>
<td>41.03 ± 12.07</td>
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<td>CS1⁺ cells</td>
<td>34.80 ± 11.70</td>
<td>39.19 ± 15.68</td>
<td>0.2237</td>
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</table>

Data are mean ± standard deviation. Significant p values are indicated in bold.
FIGURE 2.9 Comparison of the proportion of 2B4-expressing cells in gated populations from PBMCs between SLE patients (SLE) (n=45) and healthy controls (HC) (n=30). (a) Lymphocyte and monocyte populations were gated based on forward and side scatter. (b) T cells stained with anti-CD3-FITC were gated. (c) NK cells stained with anti-CD56-APC were gated. (d) Monocytes stained with anti-CD14-FITC were gated. For each gate, the proportion of 2B4-expressing cells was calculated by subtracting the percentage of PE-conjugated mouse IgG1-stained cells from the percentage of PE-conjugated anti-2B4 antibody-stained cells. Asterisk indicates significant differences between HC and SLE groups.
FIGURE 2.10 Mean fluorescence intensity ratio (MFIR) of cells positively stained for 2B4 from patients with SLE (n=45) vs. healthy controls (n=30). (a) Total 2B4-positive cells were gated within lymphocyte and monocyte populations, which were identified by forward and side scatter. (b) 2B4-positive cells were gated within CD3-positive cells. (c) 2B4-positive cells were gated within CD56-positive cells. (d) 2B4-positive cells were gated within CD14-positive cells. MFIR was calculated by dividing the mean fluorescence intensity (MFI) of samples with MFI of isotype controls. Asterisk indicates significant differences between HC and SLE groups.
FIGURE 2.11 Comparison of the proportion of CS1-expressing cells in gated populations from PBMCs between SLE patients (SLE) (n=40) and healthy controls (HC) (n=30).  (a) Total lymphocyte population was gated based on forward and side scatter. (b) T cells stained with anti-CD3-FITC were gated. (c) NK cells stained with anti-CD56-APC were gated. (d) B cells stained with anti-CD19-APC were gated. For each gate, the proportion of CS1-expressing cells was calculated by subtracting the percentage of PE-conjugated mouse IgG2a-stained cells from the percentage of PE-conjugated anti-CS1 antibody-stained cells. Asterisk indicates significant differences between HC and SLE groups.
FIGURE 2.12 Mean fluorescence intensity ratio (MFIR) of cells positively stained for CS1 from patients with SLE (n=40) vs. healthy controls (n=30). (a) Total CS1-positive lymphocytes were gated within lymphocyte population, which was distinguished from monocytes and granulocytes by forward and side scatter. (b) CS1-positive cells were gated within CD3-positive cells. (c) CS1-positive cells were gated within CD56-positive cells. (d) Total CD19-positive B cells were gated. MFIR was calculated by dividing the mean fluorescence intensity (MFI) of samples with MFI of isotype controls. Asterisk indicates significant differences between HC and SLE groups.
CHAPTER III

CS1-EXPRESSING SLE B CELLS

SUMMARY

B cells play a central role in the pathogenesis of SLE by producing pathogenic autoantibodies. Several subsets of CD19$^+$ B cells, which correlate with active disease, have been identified in PBMCs of patients with SLE. Among them, plasmablasts are short-lived antibody-producing B cells which are identified by no expression of CD20, low expression of CD19, and high expression of CD38 on the surface. In this study, we found a linear relationship between the proportion of CS1-expressing SLE B cells and SLEDAI levels. Interestingly, three distinct B cell populations were identified in SLE based on the level of CS1 expression; CS1$^{\text{negative}}$ B cells, CS1$^{\text{low}}$ B cells, and CS1$^{\text{high}}$ B cells. Further analysis of CS1$^{\text{high}}$ B cells reveal that these cells are recently described plasmablasts, displaying high expression of CD27, CD38, and HLA-DR, no expression of CD20, and the expression of plasma cell-specific transcription factor Blimp-1.
INTRODUCTION

The main effector function of B cells is to produce antibodies. Therefore, antibody-producing B cells are also called effector B cells. There are two subsets of antibody-producing B cells; short-lived, proliferating plasmablasts, and long-lived, non-proliferating plasma cells (Grammer and Lipsky 2003). As producers of pathogenic autoantibodies, SLE B cells play a central role during SLE pathogenesis. Abnormal functions of SLE B cells include reduced threshold level for BCR activation, overproduction of anti-inflammatory cytokine IL-10, and autonomous B cell homotypic stimulation (Llorente, Richaud-Patin et al. 1993; Desai-Mehta, Lu et al. 1996; Kumar, Li et al. 2006). All these factors can promote plasma cell differentiation and dysregulation of antibody production (Wehr, Eibel et al. 2004).

Over the last years, a distinct subset of SLE B cells has been shown to associate with active disease. Initially, an increase of early plasma cells was found in the patients with active SLE, and these cells expressed high levels of CD38 and low levels of CD19 (Harada, Kawano et al. 1996). Later, these early plasma cells were named plasmablasts, which displayed high levels of surface CD27 expression and low levels of CD19 expression (Odendahl, Jacobi et al. 2000). These plasmablasts were strongly correlated with disease activity in SLE patients and sorted cells (CD20^−/CD27^{high}) spontaneously produced antibodies (Jacobi, Odendahl et al. 2003). Recently, it was shown that plasmablasts are able to be distinguished from plasma cells by the expression of HLA-DR; plasma blasts are HLA-DR^{high}/CD27^{high} and plasma cells are HLA-DR^{low}/CD27^{high}.
(Jacobi, Mei et al. 2010). In addition, the percentage of B cells lacking RP105 (CD180), a negative regulator of TLR-4 signaling (Divanovic, Trompette et al. 2005), was found to correlate with active SLE, and these cells also showed a CD38$^{\text{high}}$, CD19$^{\text{low}}$, and CD86$^{\text{positive}}$ phenotype (Koarada, Tada et al. 1999; Kikuchi, Koarada et al. 2002; Koarada, Tada et al. 2010). Also, CD86-expressing B cells were reported to associate with active SLE (Bijl, Horst et al. 2001).

Furthermore, some memory B cells were also reported to correlate with SLE disease activity. Initially, the expansion of CD19$^{\text{high}}$/CD20$^{\text{low}}$ B cells was found in SLE as well as Common variable immunodeficiency (CVID) patients (Warnatz, Wehr et al. 2002). Another study found CD19$^{\text{high}}$ memory B cells from SLE patients are increased with activated phenotype and exhibit high level of antibodies against small nuclear ribonucleoproteins (Culton, Nicholas et al. 2007). These CD19$^{\text{high}}$ memory B cells were enriched in autoreactivity and correlated with adverse outcomes in SLE (Nicholas, Dooley et al. 2008). Recently, Jacobi et al. showed that CD27/IgD/CD95$^{+}$ memory B cells are correlated with active SLE (Jacobi, Reiter et al. 2008). However, whether these CD95$^{+}$ memory B cells are the same as CD19$^{\text{high}}$ memory B cells are unknown.

In this study, we investigate the relationship between SLE disease activity index with the percentage of immune cells expressing 2B4 and CS1. The proportion of CS1-expressing B cells was associated with active SLE, and distinct subsets of SLE B cells were identified based on the surface expression level of CS1. Finally, CS1$^{\text{high}}$ SLE B cells were identified as plasmablasts.
MATERIALS AND METHODS

Analysis of CS1-expressing B cells

PBMCs from patients with SLE and healthy individuals were stained with anti-CD27-FITC, anti-CS1-PE, anti-CD19-APC, and anti-HLA-DR-APC.Cy7. After gating CS1\textsuperscript{high} CD19\textsuperscript{low} cells, CS1\textsuperscript{low} CD19\textsuperscript{high} cells, or CS1\textsuperscript{negative} CD19\textsuperscript{medium} cells, the surface expression level of CD27 and HLA-DR were determined by mean fluorescence intensity. In another set of experiments, PBMCs were stained with anti-CD20-FITC, anti-CS1-PE, anti-CD19-PE-Texas Red, and anti-CD38-APC.

Sorting of CS1-expressing B cells

PBMCs from patients with active SLE and healthy individuals were stained with CD20-FITC, CS1-PE and CD19-PE Texas Red. After gating CD19-positive cells, three different subsets of B cells were sorted by Cytopia InFlux Cell Sorter; CD20\textsuperscript{negative}, CS1\textsuperscript{high} B cells, CD20\textsuperscript{positive}, CS1\textsuperscript{middle} B cells, CD20\textsuperscript{positive}, CS1\textsuperscript{negative} B cells. The purity of sorted cells was above 90%.

RT-PCR analysis for Blimp-1 expression

RNA was isolated from sorted B cell subsets using RNA isolation kit (QIAGEN). 1 ug of RNA was used to synthesize cDNA for subsequent PCR analysis. All procedures used were the same as in a previous chapter. Blimp-1 forward primer (5’-
CGAATGCCCTTCTACCCTG-3’) and Blimp-1 reverse primer (5’-GCGTTCAAGTAAGCGTAGGAGT-3’) generated 270 bps of PCR products.

Statistical analysis

Linear regression analysis and spearman’s rank was employed to find correlations between percentage of cells and SLEDAI levels. P values below 0.05 were considered statistically significant. Data were analysed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA).
RESULTS

Association of CS1-expressing B cells with SLE disease severity

Since we find a significant difference of the proportion of CS1-expressing B cells between healthy controls and patients with SLE (Figure 2.11), we performed correlation analysis to find the relationship between this population and disease activity. However, it did not reach the statistical significance because the number of patients with active SLE was not enough (data not shown). Next we performed linear regression analysis, another statistical method to determine if a relationship exists. Interestingly, it showed that the proportion of CS1-positive B cells linearly increases with increased SLEDAI score (p=0.035, R^2==11.4%; FIGURE 3.1). This finding may be most significant because SLE is a B cell-mediated autoimmune disease.

Analysis of CS1-expressing B cells in SLE

Recently, a number of different subsets of circulating B cells were reported in SLE, including naïve B cells, memory B cells, plasma cells, and plasmablasts. These cells can be identified by surface markers such as surface immunoglobulins (IgM and IgD), CD19, CD20, CD21, CD27, CD38, CD95, and HLA-DR. Interestingly, we found that CS1 expression can also identify different subsets of SLE B cells. FIGURE 3.2 shows that co-staining with CD19 and CS1 distinguishes three distinct subsets of B cells, which are CD19_{intermediate}, CS1_{negative} B cells; CD19_{high}, CS1_{low} B cells; and CD19_{low}, CS1_{high} B cells (best illustrated by FIGURE 3.2(d)). As shown in FIGURE 3.2(a)-(c), healthy
individuals had CD19\textsuperscript{intermediate}, CS1\textsuperscript{negative} B cells as a major B cell population. In contrast, most SLE patients had all three B cell populations, and all patients exhibiting high proportion of CS1-positive B cells essentially had CD19\textsuperscript{high} and CD19\textsuperscript{low} B cell populations. As shown in FIGURE 3.2 (e) and (f), some SLE patients displayed CD19\textsuperscript{low}, CS1\textsuperscript{high} B cells as their major B cell populations. Notably, as seen in FIGURE (f), one patient with active SLE (Patient 1, SLEDAI=15) displaying highest percentage of CD19\textsuperscript{low}, CS1\textsuperscript{high} B cells had very low number of CD19\textsuperscript{+} B cells, probably affected by lymphopenia. These data suggest that CS1 can be used as a B cell surface marker to differentiate different subsets of B cells.

\textit{CS1\textsuperscript{high} B cells are plasmablasts.}

Antibody producing cells can be divided into two populations; short-lived, proliferating plasmablasts and long-lived, non-proliferating plasma cells. Recent multiple myeloma studies indicate that CS1 is highly expressed by normal plasma cells as well as tumorigenic multiple myeloma cells. However, these studies did not distinguish these two antibody-producing B cell populations. First, in order to know whether CS1\textsuperscript{high} B cells are plasma cells, we decided to use CD27 as a plasma cell marker, because recent studies showed that CD27 is upregulated as B cells differentiate into plasmablasts or plasma cells. We determined CD27 expression gated on three different B cell subsets using total PBMCs from patients with active SLE and healthy individuals. As shown in FIGURE 3.3, CS1\textsuperscript{high} B cells from patients with active SLE had higher level of CD27 on the surface.
compared to healthy individuals. This suggests that CS1\textsuperscript{high} B cells are plasma cells or plasmablasts.

It was recently described that short lived plasmablasts in SLE are correlated with active SLE disease. Since their phenotype is also CD19\textsuperscript{low}, we decided to determine whether CS1\textsuperscript{high} B cells are plasmablasts or plasma cells, using CD20, CD38 and HLA-DR as differentiating markers. As seen in FIGURE 3.4, CS1\textsuperscript{high} B cells in SLE patients displayed CD20\textsuperscript{negative}, CD38\textsuperscript{high}, and HLA-DR\textsuperscript{high} phenotype. This indicates that CS1\textsuperscript{high} B cells are plasmablasts, not plasma cells. This result makes sense because CS1 is known to induce B cell proliferation, but plasma cells are not known to proliferate.

Next, we sorted out CS1\textsuperscript{high} B cells and performed RT-PCR analysis to find whether CS1\textsuperscript{high} B cells express transcription factor Blimp-1, which is expressed by plasmablasts and plasma cells. As shown in FIGURE 3.5, sorted CS1\textsuperscript{high} B cells from one healthy individual as well as one SLE patient expressed the transcription factor Blimp-1, further demonstrating that CS1\textsuperscript{high} B cells are plasmablasts.
DISCUSSION

We found a linear relationship between CS1-expressing B cells and SLE disease severity. Interestingly, distinct subsets of SLE B cells was identified based on CS1 expression level; CS1$^{\text{negative}}$ B cells (CD19$^{\text{intermediate}}$), CS1$^{\text{low}}$ B cells (CD19$^{\text{high}}$), and CS1$^{\text{high}}$ B cells (CD19$^{\text{low}}$), suggesting CS1 can be used as a marker for differentiating different subsets of SLE B cells. CS1$^{\text{high}}$ SLE B cells were identified as plasmablasts, which may produce pathogenic autoantibodies. This data suggest an important role of CS1 in SLE B cells, including CS1$^{\text{high}}$ plasmablasts. Therefore, the transcriptional regulation of CS1 in B cells was investigated in next chapter.
FIGURE 3.1 Linear regression analysis. There was a linear relationship with positive slope between the proportion of CS1-positive B cells and SLEDAI disease activity scores (p=0.035).
p=0.035
R²=0.014
FIGURE 3.2 Co-staining of CD19 and CS1 distinguishes three distinct subsets of B cells in SLE - CD19\textsuperscript{intermediate}, CS1\textsuperscript{negative} B cells (bottom square); CD19\textsuperscript{high}, CS1\textsuperscript{low} B cells (middle square); and CD19\textsuperscript{low}, CS1\textsuperscript{high} B cells (upper square). Representative dot plots from three healthy controls ((a)-(c)) and three SLE patients ((d), Patient 5, SLEDAI=9; (e), Patient 9, SLEDAI=6; (f), Patient 1, SLEDAI=15) were shown. Anti-CD56-APC and anti-CS1-PE were used to stain B cell populations in healthy control vs. SLE patient and percentage of each subset in total CD19 B cells was indicated.
FIGURE 3.3 CS₁\textsuperscript{high} expressing B cells in SLE express high level of CD27. Total PBMCs from healthy individual (A) and SLE patients with active disease (B) were first analysed by CD19 versus CS1. After CS₁\textsuperscript{high} B cells (blue dots), CS₁\textsuperscript{low} B cells (red dots), and CS₁\textsuperscript{negative} B cells (green dots) were gated, the surface expression of CD27 was shown in histogram. As seen in panel B, CS₁\textsuperscript{high} B cells express high levels of CD27 (mean fluorescence intensity is 25), indicating that they are plasma cells or plasmablasts. One representative data out of three independent experiments was shown.
FIGURE 3.4 CS1\textsuperscript{high} expressing B cells are plasmablasts. Total PBMCs from one SLE patient with active disease were stained with anti-CD20-FITC, anti-CS1-PE, anti-CD19-PE-Texas Red, anti-CD38-APC, and anti-HLA-DR-APC.Cy7, and then analyzed by flow cytometry. CS1\textsuperscript{high} CD19\textsuperscript{positive} B cells (red dots) were shown to be CD20\textsuperscript{negative}, CD38\textsuperscript{high}, and HLA-DR\textsuperscript{high}, indicating that they are plasmablasts. For CD20, CD38 and HLA-DR vs. CS1 staining, CD19-expressing B cells were gated. One out of two independent experiments was shown.
FIGURE 3.5 CS1<sup>high</sup> expressing B cells express Blimp-1 transcription factor. Total RNAs were isolated from sorted CS1-negative B cells and CS1-high expressing B cells from one healthy individual and one patient with active SLE. RT-PCR was performed to find the expression of Blimp-1 mRNA. Whereas CS1-negative B cells from a healthy control did not express Blimp-1, sorted CS1-high expressing B cells from a healthy control and a SLE patient showed good expression of Blimp-1 mRNA.
CHAPTER IV

TRANSCRIPTIONAL REGULATION OF HUMAN CS1 GENE

SUMMARY

CS1 (CRACC or CD319) is a member of the SLAM (Signaling Lymphocyte Activation Molecule) family receptors and is expressed on NK cells, CD8+ T lymphocytes, mature dendritic cells, and activated B cells. Unlike other SLAM members, CS1 shows SAP (SLAM-associated protein) independent but EAT-2 (Ewing Sarcoma associated transcript) dependent signaling in human NK cells. SAP is mutated in X-linked lymphoproliferative disease (XLP). Recently, multiple myeloma (MM), a type of human plasma cell tumor, was demonstrated to overexpress CS1 and humanized monoclonal antibody against CS1 is in human clinical trial for MM. Understanding transcriptional regulation of CS1 will help identify the specific transcription factors that control CS1 expression in normal plasma cells and MM cells. This information may be useful in developing immune based therapy for MM and other B cell-mediated autoimmune diseases.
INTRODUCTION

SLAM (Signaling Lymphocyte Activation Molecule) family receptors are broadly expressed on hematopoietic cells and play an important role in immune regulation. Six members of this family have been characterized – SLAM (SLAMF1, CD150), CD229 (SLAMF3, Ly-9), 2B4 (SLAMF4, CD244), CD84 (SLAMF5), NTB-A (SLAMF6; Ly108 in mouse), and CS1 (SLAMF7, CRACC, CD319). The extracellular domains of these receptors have two or four Ig-like domains, and their intracellular domains have tyrosine-based signaling motifs called ITSMs (immunoreceptor tyrosine-based switch motifs). One of the important features of SLAM family receptors is their recruitment of small adapter proteins, SLAM-associated protein (SAP, SH2D1A) and/or Ewing’s sarcoma (EWS)-activated transcript 2 (EAT-2, SH2D1B) in humans. SAP is a short intracellular adaptor molecule that is mutated in humans with X-linked lymphoproliferative disease (XLP). All the SLAM family receptors except 2B4 are self-ligands and exhibit homotypic interactions (Calpe, Wang et al. 2008).

Human CS1 is expressed on NK cells, a subset of CD8\(^+\) T lymphocytes, mature dendritic cells, and activated B cells. Unlike other SLAM members, CS1 transmits SAP-independent but EAT-2-dependent signaling in human NK cells, resulting in cytolytic activity toward tumor target cells. A recent CS1-knock out mice study revealed that CS1 can play both activating and inhibitory functions, depending on EAT-2 expression (Cruz-Munoz, Dong et al. 2009). Signaling molecules activated by CS1 engagement include
PLCγ, ERK1/2, PI3K, AKT, and STAT3 (Tassi and Colonna 2005; Tai, Soydan et al. 2009). In human B lymphocytes, CS1 expression is expressed at low levels by peripheral B cells. However, the expression of CS1 can be induced upon CD40-mediated B cell activation (Bouchon, Cella et al. 2001; Lee, Mathew et al. 2007). Importantly, our laboratory previously demonstrated that CS1 induces B cell proliferation and leads to the production of autocrine cytokines such as IL-14 (Lee, Mathew et al. 2007), which play a role in the development of autoimmunity (Ambrus, Contractor et al. 1995; Shen, Zhang et al. 2006; Shen, Suresh et al. 2009). Recently, CS1 was demonstrated to be an appropriate target for multiple myeloma (MM), a malignancy of plasma cells, in that CS is highly and universally expressed on MM cells while having restricted expression in normal tissues (Hsi, Steinle et al. 2008; Tai, Dillon et al. 2008; Tai, Soydan et al. 2009; van Rhee, Szmania et al. 2009). A humanized monoclonal antibody against CS1 (HuLuc53, elotuzumab) is currently in human clinical trial for MM (van Rhee, Szmania et al. 2009).

In addition, our previous study in human systemic lupus erythematosus (SLE) patients demonstrated that CS1^{high} plasma cells are associated with active disease. Interestingly, both lupus and plasmacytomas, corresponding to human MM, can be induced by intraperitoneal injection of pristane in mice (Anderson and Potter 1969; Satoh, Richards et al. 2000).

B lymphocyte-induced maturation protein-1 (Blimp-1), also named the positive regulatory domain I binding factor 1 (PRDI-BF1), is encoded by the gene termed the positive regulatory (PR) domain zinc finger protein 1 (PRDM1). It was originally
identified as a master transcription factor for plasma cell differentiation (Turner, Mack et al. 1994). Blimp-1 is known as a transcriptional repressor mainly by interacting with histone modifying enzymes, which induce a closed conformation of the chromatin (Yu, Angelin-Duclos et al. 2000).

Among SLAM family receptors, only murine and human 2B4 promoters have been analyzed (Chuang, Lee et al. 1999; Chuang, Pham et al. 2001). In this study, we analyzed human CS1 promoter and found transcription factor Blimp-1 plays a critical role in CS1 gene expression. The purpose of this study was to determine transcription start sites of CS1 mRNAs and identify transcription factors critically involved in CS1 gene expression. Understanding CS1 gene expression and regulation will help identify the specific transcription factors that control CS1 expression in normal plasma cells and MM cells. This information may be useful in developing immune based therapy for B cell-mediated diseases, such as SLE and MM.
MATERIALS AND METHODS

Cell culture and reagents

YT (human NK tumor cell line), and DB (human diffuse large B cell lymphoma cell line) cells were cultured in 4+ RPMI complete medium (RPMI 1640 supplemented with 10% FBS, 2mM glutamine, 100U/ml penicillin, 100ug/ml streptomycin, 10mM HEPES, and 10mM nonessential amino acids). NCI-H929 (human multiple myeloma cell line) cells were cultured in 4+ RPMI complete medium with 0.1mM beta-mercaptoethanol (2-ME).

Cloning of promoter region of human CS1 gene

Genomic DNA (gDNA) was isolated from 500 microliters of human venous blood using FlexiGene DNA kit (Qiagen, Valencia, CA). 50 to 100ng of gDNA was used to amplify an approximately 1.7kb upstream region of human CS1 gene by hCS1p FP (5’-GATCATGCCACTACACTCCAG-3’) and hCS1p RP (5’-TAAAGAAGCAGGAGGGGTCACG-3’). Successful PCR products were gel eluted and subjected for TA cloning into pGEM-T-easy vector (Promega, Madison, WI).

Determination of transcription initiation sites of human CS1 mRNAs

Total RNA was prepared from NK-92MI, YT, and DB cell lines using RNA-stat-60 RNA isolation reagent (TEL-TEST B, Friendswood, TX). Transcription initiation sites
were determined by RNA ligase-mediated rapid amplification of the cDNA ends (RLM-RACE) in accordance with the manufacturer’s instructions (FirstChoice RLM-RACE kit, Ambion, Austin, TX). In brief, total RNA was treated with calf intestine alkaline phosphatase (CIP) to remove free 5’ phosphates from other RNAs except capped mRNAs. Then, tobacco acid pyrophosphatase (TAP) treatment removes the cap structure from full-length mRNAs, leaving a 5’-monophosphate. Using T4 RNA ligase, a 45 base RNA adapter oligonucleotides were ligated to decapped mRNAs. A random-primed reverse transcription reaction and nested PCR amplified the 5’ ends of human CS1 transcripts. The primer sequences used for PCR reaction are followed – Outer RP : 5’-TTCTGGCTGTATGGTGACAAGAGG-3’, Inner RP1 : 5’-CTTGCTTTACTTTGGACTTC-3’, and Inner RP2 : 5’-GACCCTGTGAGCTGCCAAAGGAT-3’.

*Generation of promoter deletion mutants*

In order to measure transcription activity of CS1 promoter, CS1 promoter was subcloned into pGL3-basic luciferase reporter vector. 1.7kb of CS1 promoter region was PCR amplified using forward (5’-GATGGTACCTGCCACTACACTCCAG-3’ containing a KpnI site) and reverse (5’-GGACT AAGCTTGAGCTGCCAAAGGAT-3’ containing an HindIII site) primers, and then subcloned into pGL3-basic vector. For the purpose of identifying the transactivating or transrepressing elements in the CS1 promoter, eight 5’ deletion mutants were generated from the 1.7 kb CS1 promoter.
construct. All fragments/mutants were PCR amplified and directly cloned into pGL3-
basic vector. Successful clones were introduced into CS1-expressing cell lines by
electroporation at 200V and 950µF. 48 hours after electroporation, the promoter activity
was assessed using the Dual Luciferase Assay System (Promega, Madison, WI).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with a commercial
LightShift Chemiluminescent EMSA kit, according to the manufacturer’s instructions
(PIERCE, Rockland, IL). Sense and antisense 3’-end biotinylated oligonucleotides
corresponding from -776 to -731 relative to the transcription initiation site of CS1 were
synthesized from IDT DNA Technologies. Sense and antisense oligonucleotides were
denatured at 95°C for 5 minutes and annealed to room temperature (25°C) slowly at a
concentration of 1 pmol/µl in STE buffer (NaCl 50mM, Tris-HCl (pH 7.5) 10mM, EDTA
1mM) to generate biotinylated probe. For competitor oligonucleotides, sense and
antisense oligonucleotides were annealed at a concentration of 10pmol/ µl in STE buffer.
These concentrated probes and competitors were diluted with TE buffer immediately
before performing assay. Nuclear Extracts of NCI-H929 cells were prepared with NE-
PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer’s
instructions (PIERCE). A total of 8 µg of nuclear extract proteins were mixed with 2.5
fmol of biotinylated probe in 80mM NaCl, 20mM Tris-HCl (pH 7.5), 10% glycerol, and
3µg of poly (dI:dC) in the presence or absence of excess amount of specific or mutated
competitors, and incubated for 30 minutes on ice. Subsequently, the mixtures were subjected to electrophoresis on 4 to 6% acrylamide : bisacrylamide (29:1) gels and run in 0.5X Tris-borate-EDTA buffer at 4°C at 100 volts for 60 to 90 minutes. Positively charged nylon membrane was used for transfer of binding reactions at constant current of 380mA for 30 minutes. After crosslinking membranes for 15 minutes on a transilluminator at 312nm, membranes were developed by Chemiluminescent Nucleic Acid Detection Module and exposed to X-ray film for 20 seconds.

**Chromatin immunoprecipitation (ChIP) assay**

Cells were treated with formaldehyde and nuclei were isolated. Chromatin was sheared by enzymatic digestion. Sheared chromatin was immunoprecipitated by goat anti-Blimp-1 polyclonal antibody (C-21, Santa Cruz) or control goat antibody using the magnetic bead ChIP-IT™ express kit from Active Motif (Carlsbad, CA). DNA was isolated from the immunoprecipitated chromatin on Protein G-linked magnetic beads using directions supplied by the ChIP-IT™ kit manufacturer. The DNA was analyzed by PCR using forward primer (5’-TGAACACCCACAGGCAGAAG-3’) and reverse primer (5’TCAGGTAATATGGTTGACAG-3’).
RESULTS

Determination of transcription initiation site of human CS1 gene

In order to find a suitable model to study CS1 transcriptional regulation, three cell lines were tested for the expression of CS1 – NK92MI, YT, DB and NCI-H929 cells. As shown in FIGURE 4.1, the surface density of CS1, as estimated by mean fluorescence intensity ratio (MFIR), was significantly higher on DB and NCI-H929 cells than on NK-92MI and YT NK cell lines. DB cells are a cell line derived from a patient with diffuse large B cell lymphoma (DLBCL), and display phenotypes similar to germinal center B cells (Wanner, Hipp et al. 2006). High level of CS1 surface expression on DB cells is consistent with our previous publication (Kumaresan, Lai et al. 2002). NCI-H929 cells are a cell line derived from a patient with multiple myeloma, a malignant disease of plasma cells.

To identify the transcription initiation site of the human CS1 gene, 5’RACE analysis was performed using DB cells. 5’ ends of human CS1 mRNAs from DB cells were ligated with RNA linker, and cDNA was generated and used for PCR analysis. After second rounds of PCR amplification, 212bp of PCR products were identified (FIGURE 4.2). These bands were excised from the gel and subjected to TA cloning. Sequencing data obtained from these clones revealed a transcription initiation site of CS1 mRNA at 29 nucleotides upstream of the ATG translation start codon. Therefore, from now on, this transcription initiation site will be designated as +1. Interestingly, 5’ flanking region of
human CS1 gene has CAAT box (CAATCT, -91 to -86) and atypical TATA box (TTTAAATAT, -55 to -47), which may play a role in recruitment of transcription machineries. This is in sharp contrast to the multiple transcription initiation sites in human and mouse 2B4, which are driven by TATA-less promoters (Chuang, Lee et al. 1999; Chuang, Pham et al. 2001). This result suggests that CS1 gene expression may require a precise regulation by transcriptional machineries.

**Molecular cloning of 1.7kb upstream region of human CS1 gene**

Next, we decided to clone the putative human CS1 promoter regions to understand its transcriptional regulation. 5’ flanking sequence of the human CS1 gene was amplified from genomic DNA isolated from healthy human venous blood. Successful PCR products were gel eluted and then cloned into pGEM-T easy vector. Sequence analysis identified the presence of 1.7kb 5’ flanking region of human CS1 gene. As shown in FIGURE 4.3, sequence comparison between our clone and Gene Bank report identified nine point mutations and one point deletion in our clone within 1.7kb region of 5’ flanking sequence of CS1 gene, supporting the high polymorphisms found in CS1 gene.

In order to find putative transcription factor binding sites, the 1.7kb 5’flanking sequence of CS1 gene were analyzed by TRANSFAC (http://www.gene-regulation.com/cgi-bin/pub/database/transfac/search.cgi). Several putative transcription factor binding sites were found in the promoter of CS1 gene, such as TBP, CDXa, YY1,
IRF, FOXO4, AP1, NFAT1, BLIMP-1, AML1, LEF1, OCT1, c/EBP, and VDR (FIGURE 4.4).

**Binding of transcription factor Blimp-1 in the CS1 promoter**

A previous search of putative transcription factor binding sites within 1.7kb 5’ flanking region of CS1 gene identified one core sequence of Blimp-1 binding site (GAAAG) in -750 to -746 region of CS1 promoter. This was interesting because Blimp-1 is a master transcription factor regulating plasma cell differentiation. Also, previously Blimp-1 was shown to be expressed by CS1-high expressing B cells (FIGURE 3.5). In order to find Blimp-1-expressing cell line, RT-PCR was performed using cDNAs from CS1-expressing cell lines. Since NCI-H929 cells express CS1 as well as Blimp-1, this cell line was chosen for further study (FIGURE 4.5). First, in order to know whether Blimp-1 can bind to putative Blimp-1 binding site within the CS1 promoter, biotinylated oligonucleotides corresponding to -776 to -731 region of CS1 promoter were synthesized and annealed. The resulting probe was mixed with nuclear extracts prepared from NCI-H929 cells. As shown in FIGURE 4.6, the addition of nuclear proteins into the biotinylated probe resulted in a shifted band (second lane) compared to probe alone (first lane). This indicates that some proteins in the NCI-H929 nuclear extracts bound to the -776 to -731 region of CS1 promoter. Moreover, this binding was specific to the core sequence (GAAAG) of Blimp-1 binding site at -750 to -746 region of the probe, because addition of specific competitors, which have the exact same sequence with the
biotinylated probes, completely removed that shifted band (third lane), whereas addition of mutated competitors, in which core sequence of Blimp-1 binding site was changed from GAAAG into TGCGC, failed to block the binding of nuclear protein into the biotinylated probes (fourth lane). In addition, treatment of anti-Blimp-1 polyclonal antibodies generated another band right above the specific band (fifth lane). This appearance of supershifted band suggests that the nuclear complex binding to -776 to -731 region of CS1 promoter contains the Blimp-1 transcription factor.

In order to confirm the binding of Blimp-1 transcription factor in the CS1 promoter, chromatin immunoprecipitation analysis was performed using NCI-H929 cells. DNA-protein complexes were fixed and DNA was sheared into 100 to 200bp fragments by enzymatic method. Sheared DNA was immunoprecipitated with anti-Blimp-1 antibody or control antibody and then used as a template for PCR using primers amplifying -944 to -716 region of CS1 promoter. As shown by FIGURE 4.7, PCR using DNAs precipitated by anti-Blimp-1 as a template successfully amplified a product of 229bp, whereas PCR using DNAs precipitated by control antibody did not amplify any bands. Therefore, EMSA and ChIP assay data collectively shows that transcription factor Blimp-1 binds to a putative Blimp-1 binding site within the human CS1 promoter.

**Analysis of human CS1 promoter regions**

In order to identify the regions of the promoter that play a role in the transcriptional regulation of the hCS1 gene, we decided to perform dual luciferase reporter assay after
transient transfection of promoter constructs. A series of promoter mutants that contain successive deletions from the 5’ end were first amplified by PCR using specially designed primers (TABLE 4.2 & FIGURE 4.8a). After restriction enzyme digestion with KpnI and HindIII, gel-purified PCR products were inserted upstream of a firefly reporter gene within the pGL3 vector (FIGURE 4.8b). Transient transfections of 9 human CS1 promoter constructs revealed several important regions of the CS1 promoter that regulate transcription. Promoter regions (+92 to -405) and (-833 to -1350) were identified to have a positive effect on transcription, whereas the promoter regions (-405 to -588), (-726 to -833), and (-1350 to -1632) had a negative effect. Maximal luciferase activity was achieved with the promoter fragment (p-405) which produced almost 15-fold higher luciferase activity than the empty vector (FIGURE 4.9). These results reveal that the DNA sequences between -405 and +92 of the CS1 promoter region contain positive regulatory elements in Blimp-1-negative DB cells.

Next, we determined the transcriptional activity of CS1 promoter in Blimp-1 expressing YT cells. As shown in FIGURE 4.10, the transcriptional activity of CS1 promoter was quite different between DB and YT cells. In particular, DNA elements from -726 to -833 region were shown to have a strong positive regulatory region. Interestingly, this region contains the Blimp-1 binding site. This data suggest that Blimp-1 can act as a transactivating factor positively regulating CS1 expression.
DISCUSSION

We have cloned 1.7kb of 5’ flanking region of human CS1 gene which contains promoter elements. Transcription of CS1 mRNA starts from 29 nucleotides upstream of translation start codon ATG, possibly driven by atypical TATA box and CAAT box present in proximal CS1 promoter region. EMSA and ChIP assay data indicate that Blimp-1 transcription factor binds to CS1 promoter. DNA elements at -405 to +92 region of the CS1 promoter positively regulate CS1 expression in the absence of Blimp-1. In contrast to the well-known repressor function of Blimp-1, our preliminary study shows activating function of Blimp-1 in CS1 gene expression, which is also supported by coexpression of CS1 and Blimp-1 in plasmablasts. Since CS1 is a self-ligand, and CS1 is known to induce B cell proliferation, homotypic B cell-to-B cell interactions, especially between CS1<sup>high</sup> plasmablasts, may play an important role in B-cell mediated diseases such as multiple myeloma and SLE.
FIGURE 4.1 CS1 is expressed on human NK and B cell lines. NK-92MI and YT cell are human NK cell lines, and DB and NCI-H929 cells are human B cell lines. Surface expression of CS1 was determined by flow cytometry. Dotted histogram represents isotype control staining. MFIR is the mean fluorescence intensity ratio.
NK-92MI (NK cell line)  YT (NK cell line)  DB (B cell line)  NCI-H929 (B cell line)

MFIR=6.9  MFIR=3.2  MFIR=14  MFIR=18
FIGURE 4.2 RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) analysis of 5'end of CS1 mRNA. (a) Schematic diagram illustrating the procedures of RNA ligase-mediated rapid amplification of the 5’ cDNA ends (RLM-RACE). 5’ end of human CS1 mRNAs were ligated with RNA linker and cDNA was synthesized. Two rounds of PCR amplification using CS1-specific reverse primers generated 212bp of PCR products. (b) PCR products after first and second round of PCR were run on 1.2% agarose gel. Total RNAs from DB cells were used. 212bp of PCR products were cloned into pGEM-T easy vector and sequences were determined. Sequence analysis revealed that transcription of human CS1 gene starts from 29 upstream of translation start codon ATG.
(a) Linker -5'-' CS1 mRNA -3' 

↓

cDNA

: 1°PCR (358bp)

: 2°PCR (212bp)

(b) DB

1° 2° 100bp ladder

212bp
FIGURE 4.3 Mutations in the cloned 5’-flanking region of human CS1 gene. Cloned 5’-flanking region of human CS1 gene was sequenced and compared with the gene bank sequence AL121985.13. One nucleotide deletion (asterisk) and nine nucleotide substitutions were identified. ATG translation start codon was underlined, and transcription initiation site was marked in red. Nucleotide numbering was based on the transcription initiation site, designated by +1.
FIGURE 4.4 Position of atypical TATA box and CAAT box and putative transcription binding sites within the 5’ flanking region of human CS1 gene. Atypical TATA box was marked in red, and CAAT box was marked in blue. Several putative transcription factor-binding sites were underlined. +1 denotes the transcription initiation site identified by 5’RACE analysis.
FIGURE 4.5 Expression of transcription factor Blimp-1 in CS1-expressing cell lines. RT-PCR was performed to find a Blimp-1 expressing cell line. Correct size of PCR product (270bp) was detected in NCI-H929 and YT cells.
TABLE 4.1 Oligonucleotide sequences used for electrophoretic mobility shift assay.

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<tr>
<th>Primer</th>
<th>Oligonucleotide sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Probe FP</td>
<td>5’-GAGTGTCATGACACTTTTTCAAGTGTCgaaagCAAAAAAAAACACTGTC-3’-biotin</td>
<td>-776 to -731</td>
</tr>
<tr>
<td>Probe RP</td>
<td>5’-GACAGTTTTTTTTTTGtttcGACACTTGAAAAAGTGTCATGACACTC-3’-biotin</td>
<td>-776 to -731</td>
</tr>
<tr>
<td>Specific</td>
<td>5’-GAGTGTCATGACACTTTTTCAAGTGTCgaaagCAAAAAAAAACACTGTC-3’</td>
<td>-776 to -731</td>
</tr>
<tr>
<td>Specific</td>
<td>5’-GACAGTTTTTTTTTTGtttcGACACTTGAAAAAGTGTCATGACACTC-3’</td>
<td>-776 to -731</td>
</tr>
<tr>
<td>Mutated</td>
<td>5’-GAGTGTCATGACACTTTTTCAAGTGTCgaaagCAAAAAAAAACACTGTC-3’</td>
<td>-776 to -731</td>
</tr>
<tr>
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<td>Competitor FP</td>
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<tr>
<td>Competitor RP</td>
<td>TGACACTC-3’</td>
<td>-776 to -731</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lower case denotes a putative Blimp-1 binding site (GAAAG) and its change into TGCGC.

<sup>b</sup> Position relative to the transcription initiation site identified by 5’RACE analysis
FIGURE 4.6 Electrophoretic mobility shift assay. 2.5 fmol of 3’-end biotinylated probe corresponding from -776 to -731 (containing consensus Blimp-1 binding site) relative to the transcription initiation site of CS1 was incubated without and with 8µg of NCI-H929 nuclear extracts in first and second lane, respectively. Specific competitor has exactly same sequence with probe whereas mutated Blimp-1 competitor has same sequence with probe except mutated Blimp-1 consensus site (GAAAG -> TGCGC). Binding reaction was performed on ice for 30 minutes. Specific DNA-protein complex is indicated by empty arrow. Supershifted band is also seen when anti-Blimp-1 polyclonal antibody was added.
<table>
<thead>
<tr>
<th>Probe</th>
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<tr>
<td>Specific competitor</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Mut Blimp-1 competitor</td>
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<td>-</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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</table>
FIGURE 4.7 Chromatin immunoprecipitation assay. NCI-H929 cells were fixed by formaldehyde and chromatin was enzymatically sheared. Sheared chromatin was incubated with anti-Blimp-1 polyclonal antibody or control goat antibody. Immunoprecipitated chromatin was extensively washed and released from the beads by protease K incubation. PCR was performed using specific primers recognizing near putative Blimp-1 binding sites within CS1 promoter. Whereas goat antibody-pull down failed to produce any PCR products, anti-Blimp-1 antibody-pull down successfully amplified 229bp of PCR products after 36 cycles of PCR.
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<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>p-726F</td>
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<td>p-833F</td>
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<td>-833 to</td>
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<tr>
<td>p-1196F</td>
<td>5’-TATTCCgttgaccACACAGGATGCC-3’</td>
<td>-1196 to</td>
</tr>
<tr>
<td>p-1350F</td>
<td>5’-TAAgttgaccTATACCTAGTTCAAC-3’</td>
<td>-1350 to</td>
</tr>
<tr>
<td>p-1632F</td>
<td>5’-GATgttgaccTGCCACTACACTCCAG-3’</td>
<td>-1632 to</td>
</tr>
<tr>
<td>p+92R</td>
<td>5’-GGACTttcgaaTGAGCTGCCAAAGGA-3’</td>
<td>+92 to +67</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lower case denotes additional oligonucleotides coding for restriction sites.

<sup>b</sup> Position relative to the transcription initiation site identified by 5’RACE analysis.
FIGURE 4.8 Construction of successive 5’-deletional mutants of the CS1 promoter. (a) Each 5’ deleional fragment of human CS1 promoter were PCR amplified using the oligonucleotides shown in TABLE 4.1. The PCR bands from lane A through I represent 1.72kb, 1.44kb, 1.29kb, 0.93kb, 0.82kb, 0.68kb, 0.5kb, 0.32kb, and 0.18kb, respectively. (b) Schematics of each deleional mutants of the CS1 promoter. All PCR products were successfully cloned directly into pGL3 vector after restriction enzyme digestion with KpnI and HindIII.
FIGURE 4.9 Dual luciferase reporter assay after transient transfection of pGL3-CS1 promoter constructs into Blimp-1-negative DB cells. 2 million of healthy DB cells were electroporated with 4µg of test plasmids and 4ng of pRL3-CMV control plasmids using the Gene Pulser electroporation apparatus, and firefly and renilla luciferase activity was determined 48 hours later. Relative luciferase activity equals firefly activity divided by renilla activity.
FIGURE 4.10 Dual luciferase reporter assay after transient transfection of pGL3-CS1 promoter constructs into Blimp-1-expressing YT cells. 2 million of healthy YT cells were electroporated with 4µg of test plasmids and 4ng of pRL3-CMV control plasmids using the Gene Pulser electroporation apparatus, and firefly and renilla luciferase activity was determined 48 hours later. Relative luciferase activity equals firefly activity divided by renilla activity.
CHAPTER V

DISCUSSION

The important roles of SLAM family receptors are recognized increasingly due to their broad expression in immune cells including hematopoietic stem and progenitor cells (Kiel, Yilmaz et al. 2005). Since most SLAM family receptors are self-ligands, one important feature of these receptors is their capability to mediate both homotypic and heterotypic cell-to-cell interactions. For example, CS1-expressing B cells can interact not only with nearby CS1-expressing B cells but also with other immune cells expressing CS1 such as dendritic cells. Unlike other members of the SLAM family, the ligand for 2B4 is CD48. Nevertheless, 2B4-expressing cells can also homotypically interact with each other, because CD48 is expressed on all hematopoietic cells including 2B4-expressing cells. There are accumulating data demonstrating a critical role played by SLAM family receptors in immune regulation (Bhat, Eissmann et al. 2006; Chan, Westcott et al. 2006; Veillette 2006). In particular, recent studies indictate that extensive polymorphisms in genes encoding SLAM family receptors are strongly associated with the susceptibility to SLE (Wandstrat, Nguyen et al. 2004; Cunninghame Graham, Vyse et al. 2008; Suzuki, Yamada et al. 2008; Ota, Kawaguchi et al. 2010). However, there have been no studies on the expression of SLAM family receptors in patients with SLE. The first and second part of this study found altered expression of 2B4 and CS1 in SLE and
an association between the proportion of CS1-expressing B cells and SLE disease activity. Furthermore, CS1$^{\text{high}}$ SLE B cells were identified as recently described plasmablasts, which have been shown to correlate with active SLE disease. As these results implicate the role of CS1 in SLE, the last part of this study investigated how CS1 gene expression is transcriptionally regulated in B cells.

*Alterations of 2B4 expression in SLE*

In this study, altered expression of 2B4 in SLE is supported by three different findings. First, there were alterations in the expression ratio of 2B4-A over 2B4-B splice variant in SLE. Our previous study found that IL-2-activated NK cells only express 2B4-A and do not express 2B4-B (Mathew, Rao et al. 2009). Since 2B4-A is an activating receptor in NK cells, IL-2-activated NK cells seems to perform their main task, killing of target cells, by lowering the expression of 2B4-B. Therefore, 2B4-B expression in resting NK cells might play a role in limiting killing of target cells via interaction between 2B4-A and CD48. In this study, patient with inactive SLE tends to have stronger expression of 2B4-A over 2B4-B compared to healthy controls. Interestingly, patients with active SLE had higher level of 2B4-B, indicated by low values of 2B4-A/2B4-B ratio. In support with the recent finding of reduced killing activity by NK cells in SLE (Yabuhara, Yang et al. 1996; Green, Kennell et al. 2005; Park, Kee et al. 2009), it will be intriguing to test a hypothesis that absence of 2B4-A-only expressing activated NK cells or failure of downregulation of 2B4-B by activated NK cells will result in the inability to control
active SLE. Second, reduced surface expression of 2B4 on all 2B4-expressing cells is observed in SLE. C1.7 anti-2B4-antibody used in this study is only capable of recognizing 2B4-A, not 2B4-B. The surface expression of 2B4 on NK cells can be modulated by receptor internalization as well as reduced promoter activity (Sandusky, Messmer et al. 2006; Mathew, Vaidya et al. 2007). Since enough 2B4-CD48 interaction is required to prevent killing by NK cells (Chlewicki, Velikovsky et al. 2008), those cells downregulating surface expression of 2B4 may be at higher risk of killing by activated NK cells. This may also result in decreased proportion of NK cells found in SLE, possibly by NK cell fratricide (TABLE 2.4). Third, decreased proportion of 2B4+ NK cells and 2B4+ monocytes was seen in SLE. 2B4 is known to be constitutively expressed by all NK cells and the significance of 2B4-negative NK cells is currently unknown. Similar to NK cells, monocytes also express high level of surface expression of 2B4. There is a report showing reduced surface 2B4 expression on monocytes after LPS treatment (Romero, Benitez et al. 2004). Although the role of 2B4 in monocytes is enigmatic, it was suggested that 2B4 acts as an inhibitory receptor in monocytes preventing the production of TNF-α, a proinflammatory cytokine, which is critical in pathogenesis of rheumatoid arthritis (Gonzalez-Alvaro, Dominguez-Jimenez et al. 2006).

Alterations of CS1 expression in SLE

Although altered mRNA expression ratio between splice variants of CS1 was not notable compared to that of 2B4, one patient showed no expression of CS1-S form (SLE
17; SLEDAI=4). This might be due to high proportion (around 60%) of CS1-expressing cells among total B cells in this patient because previous study indicated that highly purified B cells only express CS1-L form, but not CS1-S form (Lee, Mathew et al. 2007).

It was found that the surface expression of CS1 is significantly upregulated by CS1$^+$ T cells whereas it was significantly downregulated by CS1$^+$ NK cells in SLE. Although the role of CS1 in T cells is unknown, increased surface expression of CS1 in SLE T cells may provide more stimulation of CS1-expressing SLE B cells. Also, reduced expression of CS1 in NK cell may participate in the inability of NK cell killing against hyperactive SLE B cells.

Most strikingly, there was a significant increase of CS1-expressing B cells in SLE compared to healthy control (FIGURE 2.12). In addition, regression analysis showed that there is a linear relationship with positive slope between the proportion of CS1-positive B cells and disease activity (FIGURE 3.1). These data provide a possibility that altered CS1 expression in B cells might be critical in SLE pathogenesis.

SLE B cells undergo active proliferation and differentiation (Bradshaw, Zheng et al. 2008). Our previous study showed that CS1 can induce B cell proliferation by increasing autocrine cytokine production. This study also showed that the expression of CS1 on B cells is induced upon CD40-mediated B cell activation (Lee, Mathew et al. 2007). Because CS1 is homophilic, it will result in further proliferation of CS1-expressing B cells. Thus, elevated expression of CS1 on B cells in SLE may enhance the B cell proliferation. In fact, we observed that B cells isolated from patients with SLE
show more proliferation in response to agonist anti-CS1 antibody than those from healthy control (data not shown). At present, we do not know whether SLE is causing the higher expression of CS1 on B cells or the elevated CS1 expression seen in B cells from SLE patients is causing the proliferation of B cells. The mechanism of CS1 gene induction is being investigated which may provide a better understanding of the CS1 function in normal and disease conditions. Critical roles of CS1 in controlling B cell proliferation is further indicated by recent multiple myeloma studies. Multiple myeloma is a human plasma cell tumor disease. CS1 is overexpressed by multiple myeloma cells and promotes cell adhesion, clonogenic growth and tumorigenicity via interactions with bone marrow stromal cells (Hsi, Steinle et al. 2008; Tai, Soydan et al. 2009). An anti-CS1 humanized monoclonal antibody has been shown to inhibit multiple myeloma cell adhesion and induce NK cell cytotoxicity against multiple myeloma cells (Tai, Dillon et al. 2008). It will be valuable to find out whether use of anti-CS1 mAb could dampen the autoantibody production by B cells in SLE patients.

**Different subsets of SLE B cells**

SLE is characterized by hyper-reactive B cells that produce pathogenic autoantibodies. However, detailed features of B cell abnormalities are largely unknown. Recently, a number of different subsets of circulating B cells were reported in SLE, including naïve B cells, memory B cells, plasma cells, and plasmablasts (Dorner, Jacobi et al. 2009). Our flow cytometry study also found distinct subsets of CD19-positive B
cells in PBMCs of SLE patients, based on CS1 expression; CS1<sup>negative</sup> B cells (CD19<sup>intermediate</sup>), CS1<sup>low</sup> B cells (CD19<sup>high</sup>), and CS1<sup>high</sup> B cells (CD19<sup>low</sup>) (FIGURE 3.3). According to recent publications, the majority of CD19<sup>+</sup> B cells are IgD<sup>+</sup> and CD27<sup>-</sup>, indicating naïve B cells (Culton, Nicholas et al. 2007). They also found CD19<sup>high</sup> B cells to be autoreactive memory B cells, and the frequency of this population correlates with disease activity (Culton, Nicholas et al. 2007; Nicholas, Dooley et al. 2008). Also, active SLE disease has been shown to correlate with high frequency of plasma cells, which express high level of CD27 and low level of CD19 (Odendahl, Jacobi et al. 2000; Jacobi, Odendahl et al. 2003). Based on these studies, we believe that CS1<sup>negative</sup>, CD19<sup>intermediate</sup> B cells are naïve B cells; CS1<sup>low</sup>, CD19<sup>high</sup> B cells are memory B cells; and CS1<sup>high</sup>, CD19<sup>low</sup> B cells are plasma cells or plasmablasts. It is noteworthy that some patients with active SLE have CS1<sup>high</sup>, CD19<sup>low</sup> B cells as their major B cell population (FIGURE 3.3). Further analysis of these CS1<sup>high</sup>, CD19<sup>low</sup> B cells revealed that this population corresponds to recently described plasmablasts by showing high expression level of CD27, CD38, and HLA-DR, no expression of CD20, and the expression of plasma cell-specific transcription factor Blimp-1 (Dorner, Jacobi et al. 2009). The high level of CS1 expression on plasmablasts makes sense because one of the known roles of CS1 is to induce B cell proliferation. It will be interesting to investigate whether fully differentiated plasma cells, which are known as non-proliferating cells, also express CS1 or not.

*Transcription control of CS1 in B cells*
In the last part of this study, the transcriptional regulation of human CS1 gene expression was investigated. When we compared the surface CS1 expression level on DB and NCI-H929 cells (human B cell lines) with that of YT and NK-92MI cells (human NK cell lines), the CS1 expression level was higher on DB and NCI-H929 cells compared to YT or NK-92MI cells. This higher expression of CS1 on B cells (especially on CS1\textsuperscript{high} B cell) is also seen in primary cells (FIGURE 2.12 and FIGURE 3.2). This is also supported by recent multiple myeloma (MM) studies, which showed that CS1 is highly expressed by normal plasma cells and MM cells compared to other CS1-expressing cells, including NK cells, NK-like T cells, a subset of CD8\textsuperscript{T} T cells, activated monocytes and mature DCs (Hsi, Steinle et al. 2008; Tai, Soydan et al. 2009).

5’RACE and subsequent sequencing analysis found that the transcription of CS1 mRNA starts from 29 nucleotides upstream of translation start codon ATG. This single transcription initiation site for CS1 transcription is in sharp contrast to the multiple transcription initiation sites in human and mouse 2B4 genes, which are driven by TATA-less promoters (Chuang, Lee et al. 1999; Chuang, Pham et al. 2001). Interestingly, CS1 promoter has CAAT box at -91 to -86 region and atypical TATA box at -55 to -47 region from the transcription initiation site. This result suggests that CS1 gene expression may require a precise regulation by transcriptional machineries.

Successful cloning of 1.7kb upstream region of human CS1 gene and its sequencing analysis found nine point mutations and one point deletion compared to the Gene Bank sequence (AL121985.13). Since I used normal Taq polymerase plus Vent Taq
polymerase which is high fidelity polymerase having 3’ to 5’ proofreading exonuclease activity, error rate should be minimal. Therefore, those mutations in our CS1 promoter clone may be derived from the original polymorphisms in the CS1 promoter region (Farwell, McMahon et al. 2004).

One of the potential transcription factors binding to the CS1 promoter region is Blimp-1, which is known as a master transcription factor for plasma cell differentiation. EMSA and ChIP assay performed on Blimp-1 expressing NCI-H929 cells showed that Blimp-1 binds to the CS1 promoter region. Our several trials for transcriptional reporter assay in NCI-H929 cells failed probably due to low efficiency of transfection in this multiple myeloma cells. However, our preliminary study using Blimp-1-expressing YT cells indicates that Blimp-1 acts as a transactivator for CS1 expression. In vitro mutagenesis of putative Blimp-1 binding site will verify whether plasma cell-specific transcription factor Blimp-1 acts as a transactivator or repressor of CS1 gene expression.

Transient transfection and transcriptional reporter assay using successive 5’deletional mutants of the CS1 promoter revealed that -405 to +92 region has the basal promoter activity of human CS1 gene in Blimp-1-negative DB cells. Because CS1 is known to be induced by treatment of PMA or CD40-activation in B cells (Bouchon, Cella et al. 2001; Murphy, Hobby et al. 2002) and NF-kB inhibitor PDTC can suppress the induction of CS1 (data not shown), one of the potential transcription factor in CS1 induction is NF-kB. Although CS1 promoter region do not have exactly matched consensus NF-kB binding sequence (GGGRNNYYCC; R is purine, Y is pyrimidine, and
N is any nucleotides), similar sequences were found in -200 to -190 region (GGGAATATACC) and -269 to -261 region (GGGAAATGC). It will be interesting to find whether these sequences play important role in CS1 induction by PMA or CD40-activation in B cells.

**CS1 may be an important target for B cell-mediated diseases**

This study found that CS1 is altered in SLE and CS1-expressing B cells may play an important role in pathogenesis of SLE. Higher expression of CS1 in plasmablasts suggest that homotypic interactions between CS1\textsuperscript{high} plasmablasts may results in increased proliferation of plasmablasts in multiple myeloma and increased production of autoantibodies in SLE. Therefore, CS1 may be an important target for multiple myeloma and SLE. For example, anti-CS1 antibody may reduce B-cell proliferation in multiple myeloma and autoantibody production in SLE. Also, it will be interesting to test whether siRNA-mediated knockdown of CS1 results in decreased B cell proliferation and antibody production (FIGURE 5.1).
FIGURE 5.1 Schematic model of the pathogenesis of MM and SLE, and possible ways to target CS1. There are three subsets of B cells; CS1\textsuperscript{negative} B cells, CS1\textsuperscript{low} B cells, and CS1\textsuperscript{high} plasmablasts (PB). Since CS1 is a self-ligand, homotypic B-B interaction will be maximal between CS1\textsuperscript{high} PBs. Homotypic interaction between CS1\textsuperscript{high} PBs induce B cell proliferation or production of autoantibodies, resulting in MM or SLE. Anti-CS1 antibody or si-RNA-mediated knockdown of CS1 may be used against these diseases.
LISTS OF ABBREVIATIONS

NK, natural killer
PRRs, pattern recognition receptors
TLRs, toll-like receptors
PAMPs, pathogen-associated molecular patterns
TCR, T-cell receptor
BCR, B-cell receptor
PBMCs, peripheral blood mononuclear cells
MHC, major histocompatibility complex
APC, antigen presenting cell
CTLs, cytotoxic T lymphocytes
DCs, dendritic cells
Ig, Immunoglobulin
TdT, terminal deoxynucleotidyl transferase
RAG, recombination-activating gene
SHM, somatic hypermutation
CDRs, complementarity-determining regions
AID, activation-induced deaminases
CSR, isotype class switch recombination
LPS, lipopolysaccharides
MZ B cells, marginal zone B cells
FO B cells, follicular B cells
PC, plasma cells
GC, germinal center
T<sub>FH</sub>, follicular helper T cells
GC-B cells, germinal center B cells
SLE, systemic lupus erythematosus
SLEDAI, SLE disease activity index
ANA, anti-nuclear autoantibody
Anti-ds DNA, anti-double stranded DNA autoantibody
Anti-Sm, anti-Smith antigen autoantibody
SLAM, signaling lymphocyte activation molecule
SAP, SLAM-associated protein
EAT-2, Ewing’s sarcoma (EWS)-activated transcript 2
ERT, EAT-2-related transducer
MM, multiple myeloma
bps, base pairs
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